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ADVANCES IN EXOSOME-MEDIATED IMMUNOTHERAPY AND DIAGNOSTICS

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Advances in exosome-mediated immunotherapy and diagnostics

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To my family

Per aspera ad astra

ABSTRACT

Exosomes are small vesicles with immune-stimulatory capacity, which can activate T cell responses in a B cell dependent manner, and therefore may serve as immune therapeutic tools. Peptide-loaded dendritic cell (DC)-derived exosomes are proven safe in clinical trials, although with limited ability to induce cytotoxic T lymphocyte (CTL) responses or prolong patient survival. Therefore, we aimed to investigate the role of exosomal MHC/peptide complexes in immune activation and explore how to enhance exosome induced immunotherapies by applying additional stimuli to the exosomes. Bone marrow-derived dendritic cell (BMDC) exosomes loaded with ovalbumin (OVA) and α-galactosylceramide (αGC) were used for this purpose. Exosomes lacking major histocompatibility complex (MHC) class I or those that were MHC mismatched were thoroughly studied *in vivo* for their ability to stimulate effector T cells and humoral responses. In addition, we applied a novel strategy, lyophilization, for exosomal loading of antigen and adjuvants. Here, OVA, CpG-ODN and αGC were added to RAW 264.7-derived exosomes and assessed for their immune-stimulatory capacity. We demonstrated that exosomal MHC/peptide complexes were redundant for T cell stimulation *in vivo* in the presence of whole OVA, as $MHCI^{-/-}$ and allogeneic exosomes could successfully induce $CD8^{+}$ T cell responses and inhibit tumor progression (study I). Importantly, allogeneic exosomes served as an adjuvant by the upregulation of T follicular helper (Tfh) cells and increased antigen-specific antibody production (study II). We also discovered that lyophilization was feasible for loading exosomes without markedly altering exosome characteristics. Notably, additional use of the TLR9 ligand CpG-ODN improved their immune-stimulatory properties and achieved tumor regression (study III).

Selective loading and accumulation of certain tissue-specific proteins and RNA into exosomes provides a platform for potential biomarker analysis, the advantages of which include the accessibility of vesicles in body fluids ("liquid biopsies"), and the ability to trace cellular origin. However, limited material often restricts exosome proteomic analyses. Therefore, we aimed at applying the highly sensitive proximity extension assay (PEA) on cell line- and body fluid-derived exosomes to investigate the potential of using PEA for exosome protein evaluation. We confirmed that PEA can be applied on exosomes to trace their cellular source and to identify accumulated vesicle proteins. Also, the protein content of the body fluid-derived exosomes from breast milk and seminal fluid displayed diverse protein profiles (study IV), suggesting the cell/tissue traceability of exosomes by PEA and motivating their future use as biomarkers.

In conclusion, this thesis provides increased understanding of the mechanisms underlying exosome-based immunotherapies and suggests the use of impersonalized exosomes and allogenicity as a possible means of enhancing their immune-stimulatory effects in a clinical setting. In addition, this thesis offers insight into novel technologies for improved exosomal loading and the use of PEA for exosome proteomic research.

POPULAR SCIENCE SUMMARY

The immune system, or “the soldiers of the body” is important to protect us from various diseases such as bacterial and viral infections, and for removing potentially dangerous cells like cancer cells. This thesis investigates “exosomes”, small lipid droplets released by all cells in the body, which are interesting to study since they can control the immune system and both start or stop immune responses.

We aimed at exploring the use of exosomes in cancer treatment, so called immune therapy, with the purpose to activate and instruct the immune system to kill tumor cells. The potential use of exosomes as cancer immunotherapy have previously been explored in clinical trials. We know that they are safe to give to patients. However, we have not yet managed to optimize the efficacy of the exosome therapies, and we still lack the full knowledge how to improve exosome-induced cancer cell elimination. Therefore, we used mouse models to learn more about how exosomes activate immune responses and how we can change them to make them more efficient. Initially, exosomal therapy was based on using the patient’s own immune cells, collecting and loading exosomes with a small protein piece “peptide” and then giving the exosomes back to the patient. We have generated scientific evidence in our mouse models suggesting that exosomes originating from one type of immune cell, the dendritic cells, can be used from other donors and does not have to come from the patient’s themselves. Dendritic cells direct immune responses and exosomes derived from these cells share this property. In addition, exosomes can also be actively loaded to deliver proteins or other molecules within the body. We also used a novel method to add stimulatory molecules to the exosomes for an enhanced immune cell activation and better responses against the cancer.

Exosomes can be found in many different body fluids, e.g. blood, breast milk and urine and are therefore easy to access. They transport information between cells and interestingly this information can control the function of the recipient cell. The exosomal cargo reflects the cell it comes from, like a tiny “mirror image” of their parental cell. However, exosome content is not always a complete copy of their origin. In fact, some exosomal transported material accumulates inside the exosomes. Exosomes are therefore exciting to study as they can provide knowledge about diseases for example cancer, and potentially serve as diagnostic and prognostic markers. In this thesis, we also examined a novel and more sensitive method to map the exosome protein content. This method can be applied in future studies with the aim to find specific disease markers so called “biomarkers”.

In conclusion, we demonstrate that we can use unpersonalized exosomes in cancer immunotherapy. This will hopefully make exosome-based treatment more efficient, and accessible, which would be beneficial for the patient. In addition, better disease markers would provide earlier and individualized treatment which will further improve the patient prognosis. These approaches are also more cost efficient for the society. The future goal would be the possibility to not only use exosomes as cancer treatment but also as preventive vaccines to avoid development of cancer.

LIST OF SCIENTIFIC PAPERS

I. Hiltbrunner S*, **Larssen P***, Eldh M, Martinez-Bravo MJ, Wagner AK, Karlsson MCI, Gabrielsson S. **Exosomal cancer immunotherapy is independent of MHC molecules on exosomes**, Oncotarget. 2016 Jun 21;7(25):38707-38717. doi: 10.18632/oncotarget.9585

II. **Larssen P**, Veerman RE, Gucluler G, Hiltbrunner S, Karlsson MCI, Gabrielsson S. **Allogenicity boosts exosome-induced antigen-specific humoral and cellular immunity and mediate long-term memory *in vivo***
In manuscript

III. Kahraman T*, Gucluler G*, **Larssen P***, Bayyurt B, Yagci FC, Gursel A, Yildirim M, Horuluoglu B, Ayanoglu C, Eldh M, Gabrielsson S, Gursel M, Gursel I. **Loading of exosomes by lyophilization result in efficient antigen delivery and functional cancer vaccines**
In manuscript

IV. **Larssen P***, Wik L*, Czarnewski P*, Eldh M, Lof L, Ronquist G, Dubois L, Freyhult E, Gallant C, Oelrich J, Larsson A, Ronquist G, Villablanca E, Landegren U, Gabrielsson S, Kamali-Moghaddam M. **Tracing Cellular Origin of Human Exosomes Using Multiplex Proximity Extension Assay**, Mol Cell Proteomics. 2017 Mar;16(3):502-511. doi: 10.1074/mcp.M116.064725. Epub 2017 Jan 22

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PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Lukic A, **Larssen P**, Fauland A, Samuelsson B, Wheelock CE, Gabrielsson S, Radmark O. **GM-CSF- and M-CSF-primed macrophages present similar resolving but distinct inflammatory lipid mediator signatures**, FASEB J. 2017 Oct;31(10):4370-4381. doi: 10.1096/fj.201700319R. Epub 2017 Jun 21

II. Sánchez-Vidaurre S, Eldh M, **Larssen P**, Daham K, Martinez-Bravo MJ, Dahlén SE, Dahlén B, van Hage M, Gabrielsson S. **RNA-containing exosomes in induced sputum of asthmatic patients**, J Allergy Clin Immunol. 2017 Nov;140(5):1459-1461.e2. doi: 10.1016/j.jaci.2017.05.035. Epub 2017 Jun 16.

III. Parigi SM, Eldh M, **Larssen P**, Gabrielsson S, Villablanca EJ. **Breast Milk and Solid Food Shaping Intestinal Immunity**, Frontiers in immunology, 2015 Aug 19;6:415. doi: 10.3389/fimmu.2015.00415, *Review*

IV. Gehrmann U, Näslund TI, Hiltbrunner S, **Larssen P**, Gabrielsson S. **Harnessing the exosome-induced immune response for cancer immunotherapy**, Seminars in cancer Biology, 2014 Oct;28:58-67. doi: 10.1016/j.semcancer.2014.05.003, *Review*

CONTENTS

LIST OF ABBREVIATIONS

αGC	α-galactosylceramide
ADCC	antibody-dependent cellular (cell-mediated) cytotoxicity
AFM	atomic force microscopy
APC	antigen presenting cell
BALf	bronchoalveolar lavage fluid
BCR	B cell receptor
BMDC	bone marrow-derived dendritic cell
BrdU	bromodeoxyuridine
CAR	chimeric antigen receptor
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T lymphocyte-associated antigen
DAMP	damage-associated molecular pattern
DC	dendritic cell
DEX	dendritic cell-derived exosomes
DLS	dynamic light scatter
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex responsible for transport
EV	extracellular vesicle
Fab	fragment antigen binding
Fas	first apoptosis signal
FasL	first apoptosis signal ligand
Fc	fragment crystalline
FcR	fragment crystalline receptor
FCS	fetal calf serum

FDA	food and drug administration
FDC	follicular dendritic cell
FOB	follicular B cell
FoxP3	forkhead box P3
GC B	germinal center B cell
GM-CSF	granulocyte-macrophage colony-stimulating factor
GvHD	graft-*vs*-host disease
GVT	graft-*vs*-tumor
HEL	hen egg-white lysozyme
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
Hsp	heat shock protein
i.p.	intraperitoneal
i.v.	intravenous
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KIR	killer cell immunoglobulin-like receptors
LFA	leukocyte function-associated antigen
LOD	limit of detection
LPS	lipopolysaccharides
MAGE	melanoma-associated antigen
MART	melanoma-associated antigen recognized by T cells
MHC	major histocompatibility complex
miRNA	micro RNA
MMM	marginal zone metallophilic macrophages
MR	mannose receptor
mRNA	messenger RNA
MSC	mesenchymal stem cell
MVB	multivesicular body

MZB	marginal zone B cell
MZM	marginal zone macrophages
NK	natural killer cell
NKT	natural killer T cell
NPX	normalized protein expression
NTA	nanoparticle tracking analysis
ODN	oligodeoxynucleotides
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PD	programmed cell death protein
PDL	programmed cell death ligand
PEA	proximity extension assay
PRR	pattern recognition receptors
PS	phosphatidylserine
RNA	ribonucleic acid
rRNA	ribosomal RNA
s.c.	subcutaneous
SEC	size-exclusion chromatography
SEM	scanning electron microscopy
SFU	spot forming units
siRNA	small interfering RNA
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TAA	tumor-associated antigen
TAP	transporter associated with antigen processing
TCR	T cell receptor
TEM	transmission electron microscopy
TEX	tumor cell-derived exosomes

Tfh	T follicular helper cell
TGF	transforming growth factor
Th	T helper
TIL	tumor infiltrating lymphocyte
TLR	toll-like receptor
TME	tumor microenvironment
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell

1 INTRODUCTION

This part serves to provide a background to the immune system in general and also more specifically on the research topic of this thesis, namely exosomes and their role in the immune system, in immunotherapy and the use of exosomes as diagnostic markers for disease.

1.1 The immune system

The role of the immune system is to protect the host from invading pathogens such as viruses, bacteria and fungi. A multitude of cells with different properties are working together with specific proteins in an organized network to prevent and clear infections. The immune response is always dependent on a balance between defending the host, and to know when to switch off the response. This balance is clearly visualized when it comes to allergies and autoimmune diseases, caused by an increased activity of the immune system or misinterpretation of the information received. After an immune activation towards a specific pathogen an immune memory will arise, i.e. long-lived antigen-specific lymphocytes, which upon re-challenge with the same pathogen will mount a more efficient response. To simplify the function of the immune system it is generally divided into two parts; the innate immunity that is quick, non-specific and lack memory and the adaptive immunity that takes longer time to respond, is highly specific and can form a long-lasting memory. Both parts are indeed dependent on each other and some of their activity is in the grey zone bridging these two (1, 2).

1.2 The innate immune system

The natural protection provided by the innate immune system is the epithelial barriers, e.g. the skin, gastrointestinal tract and lungs where naturally occurring antimicrobial proteins and specialized immune cells are localized. The strategy of the innate immunity is to express a broad range of germline-encoded so called pattern-recognition receptors (PRR), which have evolved towards recognizing conserved regions solely present on the pathogens and not on host cells. In response to microbial presence, the innate immunity will sense their foreign surface structures, termed pathogen-associated molecular patterns (PAMPs), e.g. lipopolysaccharides (LPS), double-stranded ribonucleic acid (RNA), peptidoglycans, mannose, bacterial DNA CpG oligodeoxynucleotides (ODN) and glucans (3, 4). Another recognition pathway is activated by stressed cells which release molecules, called damage-associated molecular patterns (DAMPs) that can be sensed by immune cells, e.g. phagocytes and dendritic cells (DCs) but also by epithelial and endothelial cells. Several classes of PRRs exist, one of them that is crucial for pathogen recognition is the Toll-like receptor (TLR) family. Among those, TLR4, one of the first TLRs to be discovered, is able to recognize bacterial LPS (5), TLR9 on the other hand, recognizes unmethylated CpG-ODN (6). Neutrophils and monocytes are circulating innate immune cells that can phagocytose microbes in the circulation. They are also recruited to the site of infection by adhering to the endothelium with the aid of specific adhesion markers, integrins and selectins, and migrate through the endothelium to the infected tissue site. Upon migration of monocytes into the

tissue, they can differentiate into macrophages. The tissue-resident macrophages produce cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β when they encounter microbes. This will upregulate endothelial adhesion markers E-selectin and P-selectin for recruitment of circulating leukocytes to the infected site. Furthermore, antigen presenting cells (APC) play an important role in identifying microbes and to process and present the antigens to other immune cells of the adaptive immune system. They also secrete pro-inflammatory cytokines to provide an immunological environment that will support the effector cell activity (2).

1.2.1 Natural killer cells

Natural killer (NK) cells are bone marrow-derived lymphocytes that can identify and eliminate stressed and infected cells by using enzymes, e.g. perforin and granzymes, or by induction of death signals via first apoptosis signal ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL), which will induce apoptosis (7). In addition, they also secrete interferon (IFN)-γ to stimulate macrophage phagocytosis of pathogens (8). NK cells were initially described to induce antibody-dependent cellular cytotoxicity (ADCC) without further stimulatory signals (9). As their name "natural killer" suggests, they are programmed to kill cells without receiving prior immune signals. However, they do require presence of IFN-γ and IL-15 for the killing of tumor cells (10, 11). Major histocompatibility complex (MHC) can be sensed by NK cells as they are educated to recognize self-MHC molecules. Detection of self-MHC will engage their inhibitory signals called killer cell immunoglobulin-like receptors (KIR). In contrast, the absence of MHC class I molecules on the cell surface will activate the NK cells known as the "missing-self" theory (12).

1.2.2 Natural killer T cells

Natural killer T (NKT) cells are categorized as innate immune cells despite sharing features with both NK cells and T cells, by the expression of both NK cell markers and a T cell receptor equivalent. They are important for adaptive immune responses, and can be subdivided into type I, i.e. invariant NKT (iNKT) cells or type II NKT cells. They respond to microbial lipids or glycolipids via their MHC-related molecule CD1. For instance, iNKT cells sense and respond to the glycolipid α-galactosylceramide (αGC) presented on CD1d, and are able to produce both T helper type (Th)1 and Th2 cytokines. In addition, they can be further subdivided into $CD4^+$ and $CD4^-$ cells expressing diverse cytokine profiles, whereas sharing IFN-γ secretion (13). iNKT cells have an important role in Th1 immunity as they can be stimulated by and kill tumor cells through the release of IFN-γ, to further activate other immune cells (14).

1.2.3 Dendritic cells

In 1973, Ralph Steinman was first to describe DCs as a cell type different than macrophages by expressing high levels of MHC (15, 16). MHC complexes are surface expressed proteins important for pathogen recognition by the acquired immune system. DCs are APCs with the main function to process and present antigenic peptides on their MHC molecules for inducing

T cell activation, and they therefore serve as an important link between the innate and adaptive immune system. MHC class I molecules, expressed on all nucleated cells, bind shorter peptides, while MHC class II, expressed on APCs, bind longer peptides. Another important feature of DCs is their secretion of pro-inflammatory cytokines, e.g. IFN, TNF, IL-1, IL-6 and IL-12, for leukocyte recruitment and effector cell differentiation when they encounter pathogens (17). Moreover, DCs can be found resident in tissues that are commonly exposed to pathogens, such as mucosal tissues. Upon antigen recognition, they undergo maturation by upregulation of costimulatory molecules, e.g. cluster of differentiation (CD)40, CD80 and CD86, and also expression of MHC class I and class II molecules. During DC maturation, they also migrate to the secondary lymphoid organ, for interaction and peptide presentation to T cells (16). Notably, there are many DC subsets with diverse phenotypes; surface marker expressions, gene expression and capabilities to induce immune responses (18).

1.2.3.1 Antigen uptake and presentation by DCs

DCs can take up antigens via receptor-mediated endocytosis, phagocytosis and macropinocytosis. For the receptor-mediated uptake of antigens DCs express the C-type-lectin protein family receptors, e.g. DEC205 and the mannose receptor (MR). DCs also express Fc-receptors (FcRs) for internalization of immune complexes (19). After exogenous antigen uptake, the antigens are processed for digestion into peptides. This process occurs at low pH in the endocytic compartments, where MHC class II molecules are loaded with the peptides followed by MHC/peptide transportation to the cell surface (figure 1). In contrast, endogenous antigens are degraded in the proteasome for peptide generation (figure 1). The peptides are then transported to the endoplasmic reticulum (ER), where the MHC class I molecules are produced, the transport is performed by the transporter associated with antigen processing (TAP) proteins. Peptide loading onto the MHC class I molecules stabilizes the MHC/peptide complex and allows it to be presented on the cell surface (19). Although the mechanisms for peptide loading onto MHC class I or class II molecules are highly controlled, DCs also accomplish loading of exogenous antigens onto MHC class I molecules, called "cross-presentation", which can lead to either tolerance induction or antigen-specific $CD8^+$ T cell activation, which is an important feature for immunotherapy (20, 21).

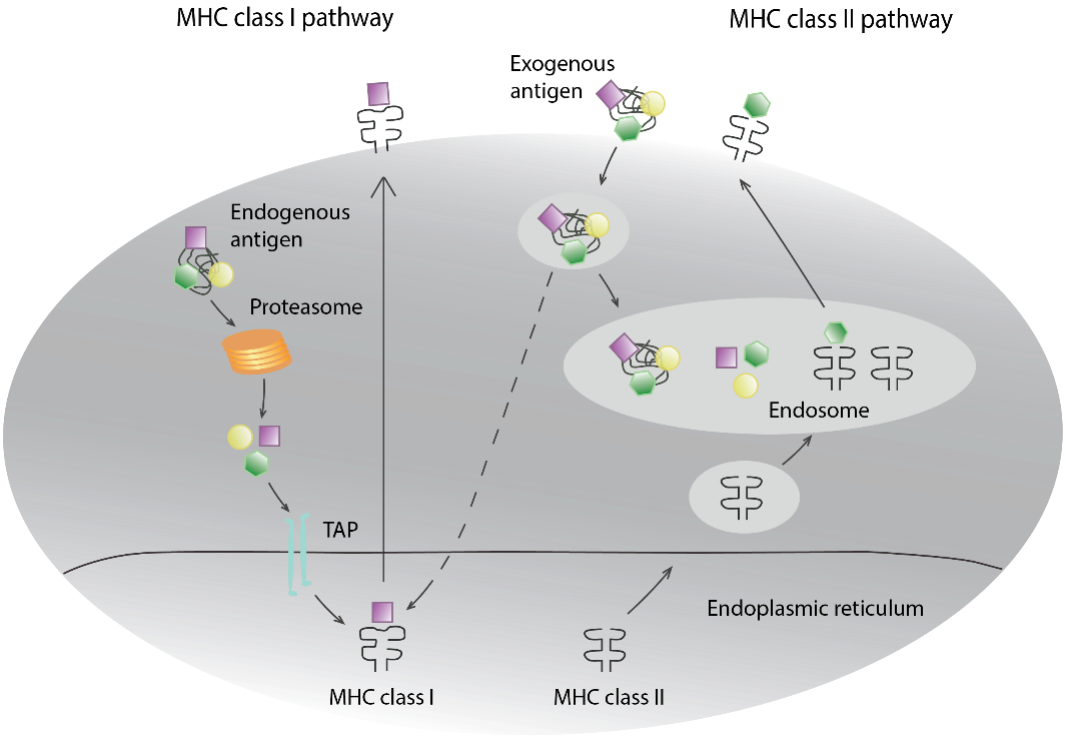

Figure 1. DCs can process antigens by different pathways for peptide loading onto MHC class I or class II molecules. For endogenous antigens, peptides are loaded on MHC class I molecules. Exogenous antigenic peptides are loaded on MHC class II molecules. DCs are also able to cross-present exogenous antigens onto MHC class I molecules.

1.3 The adaptive immune system

The adaptive immune system mediated by lymphocytes, mainly T and B cells, has a broad range of receptors for antigen recognition. The receptor diversity and specificity is mediated by somatic rearrangement of the variable regions of gene segments in a process called V(D)J recombination, where the variable (V), joining (J) and diversity (D) segments are joined together in various combinations. Thus, only a few cells will share the same antigen or peptide specificity, which gives rise to broad variability in cellular recognition of a certain peptide. Adaptive immunity is activated upon lymphocyte recognition of pathogens, which subsequently induce a long-lasting protection. Activation of an adaptive immune response with high specificity takes several days to develop, in contrast to the rapid although less specific innate immunity. B cells are responsible for the recognition of antigens and induction of a humoral response generated against many different microbe-associated proteins, carbohydrates, nucleic acids or lipids. In contrast, T cells can only identify protein fragments, peptides, presented by the APCs. Unfortunately, the adaptive immunity may also respond to antigens other than those present on microbes such as self-antigens or harmless molecules, and thus together with other cells drives diseases like autoimmunity and allergies (1).

1.3.1 Initiating an immune response

The primary lymphoid organs, bone marrow and thymus, are responsible for the production of lymphocytes, which originate from hematopoietic stem cells. Thereafter, the lymphocytes are trained and the immune responses are generated in the secondary lymphoid organs, e.g. spleen

and lymph nodes, which are strategically distributed throughout the body. The spleen is central when it comes to initiating an immune response against a pathogen, as both innate and adaptive players are present. Blood is filtered through the spleen (red pulp), allowing immune recognition of foreign antigens by APCs, followed by the interaction with lymphocytes to mount the adaptive immune response. In the lymphoid compartment of the spleen (white pulp), B and T cells are separated into different sites. B cells are located in follicles (B cell zone), which are surrounded by T cells (T cell zone). The marginal zone, between the red and the white pulp, contain resident cells with diverse functions; marginal zone macrophages (MZM) important for virus clearance, marginal zone metallophilic macrophages (MMM) that phagocytose microbes and also marginal zone B (MZB) cells, which upon pathogen recognition will produce low-affinity antibodies. Taken together, upon antigen capture an immune response is initiated, where APCs will activate naïve $CD4^+$ T cells in the T cell zone. B cells will meet the educated T cells and they interact at the border between their compartments for immune activation (22).

1.3.2 T lymphocytes

T cells are generated in the bone marrow and mature in the thymus, where they undergo a strictly controlled two-step selection for antigen specificity; i) recognition of MHC complexes (positive selection), and ii) elimination of strong MHC self-recognition (negative selection) (23). Naïve T cells are frequently scanning the secondary lymphoid organs for recognition of antigenic peptides presented on MHC class I or class II by APCs, mainly DCs. Each T cell has a T cell receptor (TCR) with specificity towards a limited set of epitopes. Upon TCR recognition of the MHC/peptide complex and subsequent recognition of the MHC class I or class II molecule by the CD8 or CD4 co-receptor, respectively, a second signal is required for T cell activation. This occurs through costimulatory receptor CD28 interaction with its ligands CD80/86 on the APC. The antigen-specific T cells undergo clonal expansion and migration to the infectious site to exert their effector functions. In brief, peptides presented by MHC class I are recognized by $CD8^+$ T cells also called cytotoxic T lymphocytes (CTL), which are specialized killer cells. Upon activation and stimulatory signals induced by $CD4^+$ T cells, CTLs will release perforin and granzymes to induce apoptotic signals that will kill the infected cell. MHC class II peptides presented by professional APC, i.e. DCs, macrophages and B cells, are recognized by $CD4^+$ T cells, which in turn can activate infected macrophages to eliminate the pathogen and induce antibody production by B cells to promote a humoral response (24). During a bacterial infection, antigens internalized by APCs from the extracellular environment will be processed and presented by MHC class II for the activation of $CD4^+$ T cell and subsequent B cell antibody production will occur. In contrast, $CD8^+$ T cell activation occurs when cytosolic antigens are present i.e. during a viral infection, by the recognition of MHC class I presented peptides (figure 1). Moreover, $CD4^+$ T cells can be further subdivided into; Th1, Th2, Th17, and regulatory T cells (Tregs), among others, based on their diverse cytokine and transcription factor profiles. Tregs are important for induction of peripheral tolerance i.e. maintaining immune homeostasis by secretion of the anti-inflammatory cytokines IL-10 and transforming growth factor (TGF)-β for inhibition of effector T cells. In addition, they are

characterized by expression of the forkhead box P3 (FoxP3) transcription factor and the surface receptors CD25, cytotoxic T lymphocyte-associated antigen (CTLA)-4 and programmed cell death protein (PD)-1, among others. Consequently, reduced activity of Tregs has been linked chronic inflammatory diseases and autoimmunity (25).

1.3.3 B lymphocytes

B cells originate from and mature in the bone marrow. They undergo positive and negative selection, according to the same principles as described for T cells, and V(D)J recombination of the heavy and light chains to generate diverse B cell receptors (BCR) (26, 27). After successful receptor editing, mature B cells express both immunoglobulin (Ig)M and IgD required to exit the bone marrow and migrate to the spleen for further maturation. The primary function of B cells is to produce high-affinity antibodies, which can neutralize and opsonize pathogens for elimination by phagocytes. B cells can be activated in a T cell-dependent manner, where B cells recognize and internalize the antigen and present it on their MHC class II molecules, thus serving as APCs. This will lead to B cell downregulation of CXCR5 and upregulation of CCR7 for migration towards the T cell zone. The signal for B cell activation also comes from the interaction with T follicular helper (Tfh) cells that have already encountered the same antigen. These are $CD4^+$ T cells, either naïve or previously differentiated T cells, which have downregulated CCR7 and upregulated CXCR5 for migration towards the B cell zone. T cell recognition of the MHC class II peptide presented by the B cell is important for B cell activation, generation of memory B cells and high-affinity antibody production (28). The majority of the B cells are located in the secondary lymphoid organs where the germinal center formation occurs. Here, the antigen-specific B cells undergo clonal expansion, Ig isotype class switching and affinity maturation. With enzymatic help, somatic hypermutation induce point mutations in the variable region for affinity maturation, and different antibody subclasses are generated through editing of the constant region of the heavy chains (29). Follicular B (FOB) cells are the majority of B cells in lymphoid organs. They undergo class switch and can give rise to long-lived plasma cells. Another activation pathway is induced by polymeric antigens such as carbohydrates in a T cell-independent manner, without the help from Tfh cells, the B cells secrete IgM antibodies and no class switching will occur. The innate-like MZB cells, located in the marginal zone respond to blood-derived antigens in a T cell-independent manner (30). Moreover, B cells are assisted by follicular dendritic cells (FDC) for antigen presentation and antigen-driven selection of high-affinity B cells, although not via MHC molecules but through complement or FcRs (31). In addition, the Tfh cells, a specific subclass of $CD4^+$ T cells previously activated by DCs, support the selection of high-affinity germinal center B (GC B) cells by providing survival signals and promote antibody production, these cells may enter the circulation to become long-lasting antibody secreting plasma cells (32).

1.3.4 Antibodies

Antibodies, also called immunoglobulins, are proteins that occur both as membrane bound BCRs on B cells or as secreted forms. Thus, a major function of B cells is to produce antibodies

that can eliminate pathogens (2). The antibody itself consists of two heavy- and two light chains, both having a constant (C) and a variable (V) region, where the variable regions together create the antigen recognition part, the fragment antigen binding (Fab). The residual part of the antibody is the fragment crystalline (Fc) region responsible for the biological activity of the antibody as the cells have Fc receptors (FcR) for antibody binding (33). Overall, there are five Ig classes; IgM, IgD, IgA, IgE, and IgG with diverse properties (33). IgM is the first antibody produced, which can eliminate pathogens in the early immune response. IgD can stimulate immune cells such as basophils and mast cells. Both IgM and IgD are expressed on naïve B cells. IgA is important for toxin and microbe elimination and is mainly found in mucosal tissue sites, e.g. the gastrointestinal tract. IgE is important in the defense against helminths, but is also linked to acute allergic responses. IgG is the primary isotype found in blood, which is important for opsonization and subsequently elimination of pathogens by macrophage phagocytosis or induction of ADCC mediated killing by NK cells. The group of IgGs can be further divided into several subclasses in humans; IgG1, IgG2, IgG3 and IgG4, and in mice; IgG1, IgG2a, IgG2b, IgG2c and IgG3, all with different effector functions (34).

1.4 Tumor immunology

The immune system is central in controlling and regulating cancer development, by the recognition of tumor cells, inhibition of tumor progression by tumor cell killing and to avoid the spreading of escaping tumor cells. In contrast, in the tumor microenvironment (TME) itself, immune cells often support the tumor growth by selecting tumor cell survival and thus inevitably driving tumor progression (35). Paul Ehrlich was first to discuss the role of cancer in relation to the host immunity in 1909 (35, 36). Since then, based on the evidence of host immunity against tumor antigens, the immunological control of cancer has been extensively investigated (37). However, the term “immunosurveillance”, i.e. the immune control of the tumor, was questioned until the 1990´s, when IFN-γ was shown important for the control of tumor establishment (38). In an immunocompetent individual, the tumor environment is constantly edited by the immune system, which will lead to the selection of resistant tumor cells that are able to cope with the hostile environment (39). Immunoediting can be divided in three stages; i) immune recognition and elimination of tumor cells, ii) controlled tumor growth balanced between the tumor and the host, and iii) tumor transformation and escape of host detection (35, 40, 41). Evidently, immune recognition of tumor and action towards elimination is linked to patient prognosis, for example tumor infiltrating lymphocytes producing IFN-γ and TNF-α is favorable for inhibiting tumor progression (38, 39, 42). Tumors may express epitopes that are different from those present on healthy cells, these are called tumor antigens or “neoantigens” (43). These proteins are transformed (mutated) or overexpressed cellular antigens (35). Neoantigens may serve as targets when designing novel treatment strategies as they have been identified for several malignancies, although, varying mutation rates thus making treatment development more or less challenging (44, 45).

1.5 Cancer immunotherapeutic approaches

This part serves to give a brief overview of some immunotherapeutic approaches currently available. Immune therapy against cancer aims to enhance or reactivate the host's immune system in order to recognize and control tumor progression. Several immune therapeutic approaches have been tested, among them monoclonal antibodies, adoptive transfer of immune cells, immune-checkpoint inhibitors and engineered immune cells. The use of tumor-associated antigens (TAA) as targets, which are native antigens overexpressed by or mutated within the tumor, is the most common method in cancer vaccination strategies and has been applied in many different cancers. Key factors in the generation of successful tumor vaccines are their potency to use TAA-restricted MHC class I peptides for the activation of $CD8^+$ T cells (CTLs) and NK cells for tumor cell elimination (46).

1.5.1 Cell-based therapies

1.5.1.1 NK cell therapy

As previously described, NK cells are able to detect and eliminate tumor cells without requiring previous activation signals. The Fc receptor CD16 on NK cells, can bind to the Fc portion of IgG to induce ADCC. Furthermore, induction of CTLs and DC maturation, which are crucial in tumor clearance, is highly dependent on innate signals, for example those derived from NK cells (47). Adoptive cell therapies using NK cells have been evaluated for both solid tumors and hematological malignancies. In colorectal cancer patients, an increased NK cell tumor infiltration was associated with a favorable prognosis, thus highlighting the significance of these cells in cancer immunosurveillance (48). In brief, NK cell therapeutic strategies focus on; i) NK cell activation, ii) hematopoietic stem cell transplantation (HSCT) (bone marrow replacement), or iii) adoptive transfer of NK cells. Thus, NK cell therapies have primarily been successful in treating hematopoietic malignancies (49). After allogeneic, T cell depleted, HSCT, NK cells mediate a graft-*vs*-tumor (GVT) effect in which transplanted NK cells recognize the host tumor cells as foreign and prompt tumor cell elimination. Importantly, the use of allogeneic NK cells did not induce graft-*vs*-host disease (GvHD), but selectively targeted the tumor cells (50, 51).

1.5.1.2 NKT cell therapy

iNKT cells can be activated directly by tumor cells expressing lipid antigens on the cell surface, which will stimulate innate and adaptive responses by the release of IL-4 and IFN-γ. Their activation provides secondary induction of NK cell activity by secretion of IFN-γ (1). The discovery that αGC could be used as a potent antigen to stimulate iNKT cell anti-tumor activity lead to the initiation of several clinical trials. However, repeated administration of αGC was unfortunately shown to induce iNKT cell anergy and little or no therapeutic effect has been demonstrated (14, 52-54). Tumor cells avoid recognition by the immune system via downregulation of CD1, whereupon they become undetectable to iNKT cells (55). In contrast to the anti-tumor role of iNKT cells, the opposite is true for the type II NKT cells, which perform immune suppression and inhibit the activity of iNKT cells (56).

1.5.1.3 DC therapy

Autologous DCs cultured *ex vivo* have been commonly used in cancer vaccines to present TAA peptides mainly for activation of $CD8^+$ T cell-mediated tumor cell killing (46). An advantage of using DCs is their capability to activate both $CD4^+$ and $CD8^+$ T cells (57). One of the first clinical trials using DC-based vaccines was in melanoma patients using autologous DCs pulsed with the MHC class I-restricted melanoma-associated antigen (MAGE)-1 peptide for induction of peptide-specific CTLs (58). Since then, many clinical trials using DCs have been performed (59, 60). Another clinical trial used the full length melanoma-associated antigen recognized by T cells (MART)-1-loaded onto DCs in the treatment of melanoma patients and identified antigen-specific $CD4^+$ and/or $CD8^+$ T cells in nearly half of the patients (61). However, the efficiency of these vaccines depends on a multitude of factors, primarily the activity level of the host immune system, the cancer state of the patient leading to immune evasion, treatment dose and the injection route. Taken together, in many cases these and other factors lead to poor clinical response to the vaccines (62). Furthermore, clinical trials using tumor lysate loaded DCs or APCs have also been tested and reported to induce some $CD8^+$ T cell responses in patients with melanoma (63) and fibrosarcoma (64). Novel DC vaccine strategies have focused on improving cell culture conditions for the production of more immune-stimulatory DCs (65, 66). Importantly, an efficient DC vaccine requires the transport of antigens to the host immune cells for an efficient $CD8^+$ T cell priming (67).

1.5.1.4 T cell therapy

T cell-based immunotherapies rely on the expansion of patient-derived tumor-specific T cells *ex vivo*, commonly both $CD4^+$ and $CD8^+$ T cells, so called adoptive T cell therapy (68). In a recent clinical study, adoptive transfer of tumor infiltrating lymphocytes (TILs) with peptide specificity for mainly TIL-3775 and TIL-3853, was used in cervical cancer patients, which demonstrated both antigen-specific $CD4^+$ and $CD8^+$ T cell responses to these peptides (69). However, TIL treatment specific for certain TAA has mainly been successful in melanoma treatment. In addition, genetically engineered T cells, chimeric antigen receptor (CAR) T cells have also been extensively tested in clinical trials (68). CAR T cells have antibodies fused to the T cell receptor which circumvent the need of tumor cell presented MHC/peptide complexes for T cell activation. Many clinical trials using genetically modified autologous T cells have been explored in the treatment of B cell malignancies, where CD19-specific CAR T cells have been used to target B cells (70). This treatment leads to an overall B cell depletion and consequently may cause severe side effects, primarily cytokine release syndrome and neurotoxicity.

1.5.2 Additional therapies

1.5.2.1 Peptide-based vaccines

Peptide-based cancer vaccines commonly aim at $CD8^+$, and not $CD4^+$, T cell stimulation, mainly using short peptides for DC loading. Limited effects have been observed in clinical

studies even in the presence of adjuvants for further enhancement of the immune response. Although these vaccine strategies were shown promising in mouse models, not all patients express human leukocyte antigen (HLA) molecules that are able to present the TAA peptides, thus excluding vaccination as an option for certain patients. Another alternative to overcome the limited effect by using solely MHC class I-restricted peptides is the use of long synthetic peptides that are able to stimulate both $CD4^+$ and $CD8^+$ T cells (46). Notably, it has been shown that long synthetic peptides, compared to whole proteins, are more efficiently processed by DCs, leading to a more efficient T cell stimulation (71).

1.5.2.2 Antibody-based therapeutics

The mechanism of action for monoclonal antibody-based tumor cell killing is mainly by induction of ADCC or complement-dependent cytotoxicity (CDC) (72). Antibodies for immunotherapy have been established and approved; the first ones being the anti-CD20 antibody used in the treatment of non-Hodgkin lymphoma (73, 74) and anti-HER2/neu antibody for the treatment of breast cancer (75). Another antibody-based therapeutic approach is checkpoint blockade therapies, such as anti-CTLA-4 and anti-PD-1 antibodies, for releasing the immunological brake induced by the TME, with the aim to reactivate immune cells in the fight against the tumor (76, 77). Notably, the full mechanistic effects of these treatments are not yet clarified, but both tumor cells and immune cells can express these molecules, and be directly targeted by the treatment. It has been demonstrated that anti-CTLA-4 treatment enhances effector $CD4^+$ T cell activity and downregulates Treg function (78, 79). Similarly, anti-PD-1 induces effector T cells, NK cells, and reduces Treg-induced immune suppression (78, 80). Both anti-PD-1 and anti-CTLA-4 have been efficient in cancer treatment and were recently approved by the US Food and Drug Administration (FDA). Unfortunately, checkpoint blockade often cause systemic adverse effects, such as inflammation, as they disrupt normal immune homeostasis. Moreover, long-term effect of such treatments are still not known (76). Another restrictive aspect of checkpoint inhibitors is that the tumor cells quickly adapt to the environment by downregulating the targeted markers, which decrease treatment efficacy.

Adaptive therapies are considered costly, time consuming and are restricted to some malignancies. Importantly, combination therapies merging several of the above-mentioned strategies are currently being tested in the treatment of cancer. One such strategy was recently tested in a clinical phase I study in melanoma patients, where TILs were infused together with DCs loaded with tumor lysate. The study demonstrated safety, albeit with limited tumor regression probably related to the low number of participants (81). Also, a combination of tumor cells and DC hybrids showed induction of CTL and tumor regression in renal cell carcinoma patients (82). Furthermore, allogeneic pro-inflammatory DCs have been intratumorally injected in patients with renal cell carcinoma and were shown to induce an anti-tumor response (83). In conclusion, passive immunotherapies based on antibodies or T cells mainly induce weak immune responses and restricted memory T cell formation and therefore provide a limited vaccine effect. Instead, active therapies for example mediated by DCs, which

drives both effector and memory T cell formation would have the potential to improve the vaccine properties.

1.6 Extracellular vesicles

1.6.1 Introduction to extracellular vesicles

All cells release extracellular vesicles (EVs), which can be found in all body fluids. They are generally subdivided into apoptotic bodies, microvesicles and exosomes, all of which are suggested to have diverse functions (84). EVs constitute a highly heterogeneous group and are commonly classified based on cellular origin, size and their different properties (85). The nomenclature is still not consistent in the vesicle field, and the functional aspects related to vesicle size are currently under investigation (86). When EVs, primarily exosomes, were discovered they were considered as a way for cells to remove unwanted material (87, 88). Today, EV research is a field under continuous expansion and some of these aspects will be further addressed in this thesis.

1.6.2 The discovery of extracellular vesicles

Extracellular vesicle release was first described for membrane vesicles of two sizes, a larger and a smaller population both carrying ecto-enzymes (89). This was followed by other studies of EVs budding off from the plasma membrane. They were also, early on, described as released secretory granules present in semen (90) and human platelet microparticles found in serum and plasma (91). Small vesicles are formed in a structured way in the multivesicular bodies (MVBs) and are subsequently secreted as vesicles (exosomes) to the extracellular environment by fusion of the MVB and the plasma membrane (88, 92). The term "exosomes" was first used for their identification during the process of transferrin receptor removal by maturating reticulocytes (93). The discovery of exosome secretion by immune cells; B cells (94), T cells (95) and DCs (96) further raised the interest to study their role in the immune system.

1.6.3 Exosome formation and secretion

All cell types secrete exosomes (97) and they have been found in different body fluids, for instance, breast milk (98), sputum (99), urine (100) and plasma (101). Exosome formation is initiated by a plasma membrane invagination and the endocytosis of cell surface proteins, which will lead to the formation of early endosomes (figure 2). Proteins can then either be recycled back to the cell surface or the early endosome can mature into late endosomes (102), which will further develop into MVBs (103). Exosomes are formed by an inward budding of the endosomal membrane (103). Hereafter, the MVBs may either be degraded by the lysosome or they can fuse with the plasma membrane to release the exosomes to the extracellular environment (31). The generation of exosomes requires packaging of proteins, lipids and other cargo, in a process guided by the endosomal sorting complex responsible for transport (ESCRT), which includes the ubiquitination, i.e. tagging of proteins for degradation (104). The ESCRT complex involves several ESCRT proteins, specifically ESCRT-0, ESCRT-I, ESCRT-

II, ESCRT-III and VSP4, which are responsible for the stepwise sorting of ubiquitylated proteins into intraluminal vesicles (105). MVBs of two different types may occur, those that are tetraspanin- and cholesterol-enriched, and those that are low in cholesterol yet high in lysobisphosphatidic acid (102). In addition, the formation of CD63 loaded exosomes was also shown to occur in an ESCRT-independent pathway, when the ESCRT-complex was silenced (106), a process that instead rely on ceramide presence (85, 107). Furthermore, proteins of the Rab family, small Ras-like GTPases, are important for intracellular transport pathways within the cells and the release of exosomes, which was demonstrated by the generation of certain Rab protein knock-outs. Importantly, the loss of Rab27a and Rab27b (108) and Rab7 (109) expression strongly reduced the release of exosomes. The Rab proteins are not continuously expressed, suggesting that different cell types develop their own vesicle release pattern (85). For exosome release, the MVBs fuse with the plasma membrane, a process suggested to be mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (85). Exosomes derive from the endosomal pathway and are generally considered to be 30-150 nm in diameter and float at a density of 1.13-1.19 g/ml. Microvesicles, on the other hand, are roughly 100-1000 nm in size (110) and depending on definition, float at a density around 1.12-1.21 g/ml (111). They bud off directly from the cell surface, a process that was first described to occur in platelets (112), and early described upon neutrophil stimulation (113). Further, apoptotic bodies, roughly 1000-5000 nm sized vesicles, are generated when cells undergo programmed cell death as an organized form of packing their cellular compartments for elimination (19, 114, 115).

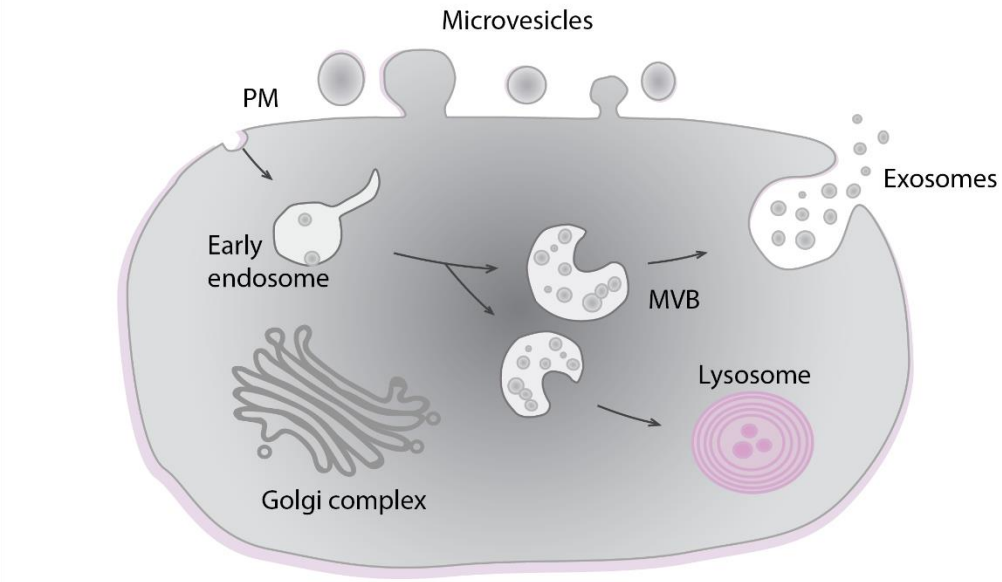

Figure 2. Exosome biogenesis starts by endocytosis of proteins at the plasma membrane and the formation of early endosomes. During late endosome maturation, intraluminal vesicles are formed by inward budding of the limiting membrane, which give rise to MVBs that can fuse with the lysosome for degradation or be transported to the plasma membrane for fusion and exosome release. Microvesicles on the other hand directly bud off from the plasma membrane.

1.6.4 Exosome composition

Exosomes are built up by a lipid bilayer containing cholesterol, phosphatidylserine (PS) and sphingomyelin (116). They also contain a large diversity of proteins, many of which are common to the majority of exosomes (117). Such proteins are those involved in the MVB formation of exosomes, for example the ESCRT proteins. Others are related to the membrane transport and lastly some of them are important for the fusion with the plasma membrane, as described for the Rab proteins (108, 118). Exosomes are, compared to the cell membrane, commonly enriched in tetraspanins, such as CD9, CD63 and CD81, a group of proteins involved in exosome formation and protein sorting onto exosomes (117, 119). Moreover, tetraspanins are also central in the regulation of ESCRT-independent vesicle formation (85). Tetraspanins are transmembrane proteins involved in various biological functions, e.g. cell adhesion and membrane fusion, but besides being present on exosomes they are also expressed on other vesicle subtypes (120). Different vesicle populations were compared for their tetraspanin content, which revealed that CD9, CD63 and CD81 positive vesicles represented exosomes, while solely CD9 was present on plasma membrane associated vesicles, i.e. microvesicles (111). In addition, ESCRT proteins and some of their associated proteins Alix and VSP4B can also be found on exosomes. Also, Syndecan was shown to recruit Alix and Syntenin and support their essential interaction for intraluminal budding of the endosomal membrane, a function that is blocked in the absence of Rab7 (109). Furthermore, exosomes contain Tsg101 and Annexin, proteins that are involved in the docking of MVBs with the plasma membrane to secrete the exosomes (97). Exosomes are also enriched in heat shock proteins (Hsp), e.g. Hsp70 and Hsp90 (117). In general, exosomal protein content is believed to be reflective of their cell of origin, on the health status of the cell and the stimuli that induced exosome secretion (85) (figure 3). For example, exosomes from APCs, e.g. DC-derived exosomes (DEX), carry MHC class II molecules and other DC related markers such as costimulatory molecules (96). Interestingly, exosomes also carry messenger RNA (mRNA) and micro RNA (miRNA) located inside the exosomes safely protected from degradation by RNases for transportation to the recipient cell (121). The RNA is believed to be packed into the exosomes in a strictly regulated manner (122). Mainly small RNA, with a low molecular weight, can be detected in exosomes. In contrast, ribosomal RNAs (rRNA) (18S and 28S) are generally absent in exosomes (123). However, it was recently shown that some exosome fractions may as well contain 18S and 28S rRNA subunits (124), thus the exosomal RNA content observed might be influenced by the isolation technique used and purity of the vesicle preparations. Interestingly, exosomal RNA content is not entirely reflecting their cell of origin, which points to a selective loading of certain RNA into the exosomes (121, 122, 125).

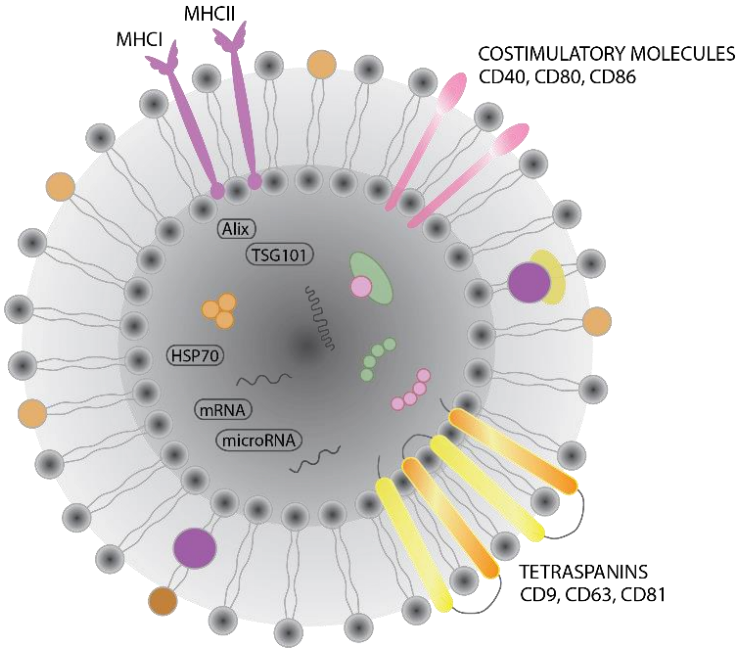

Figure 3. Illustration of the molecular composition of dendritic cell-derived exosomes carrying surface markers, e.g. tetraspanins, costimulatory molecules, MHC class I and class II and also mRNA, miRNA and other proteins involved in vesicle formation.

1.6.5 Exosome isolation and characterization

1.6.5.1 Exosome isolation methods

There are several ways to isolate extracellular vesicles from cell supernatant and biological fluids. The choice of isolation method depends on the amount of available material, experimental design, type of exosomes, their origin (cell cultures or body fluid) and the specific research question (126, 127). One of the most commonly used methods for exosome isolation is differential centrifugation (93, 128), where vesicles of different size and density are pelleted based on centrifugal force. In brief, cells and debris are discarded at a centrifugation force of 300x g, followed by removal of large protein aggregates and apoptotic bodies at 3,000x g. Thereafter, larger vesicles like microvesicles are isolated at around 10,000x g, followed by exosome isolation at around 100,000x g, even though protocols might vary slightly. Size restricted filtration may be used to remove larger particles between the 10,000x g and the 100,000x g spin. In addition, the samples may be further purified using a density gradient, e.g. sucrose gradient or iodixanol (111, 128). Moreover, exosome isolation kits can be used for quick vesicle isolation, such as precipitation, bead-based or immunoaffinity-based methods. However, unknown isolation buffers and conditions might alter exosomal characteristics by these procedures, which may affect downstream applications. In addition, the precipitation will co-pellet all vesicles, without selecting diverse vesicle populations based on size. Another commonly used isolation method is size-exclusion chromatography (SEC). Vesicles interacts with beads in a column for separation based on size and not on molecular weight, providing a gentle separation leaving the exosomes unaffected (129). Importantly, the isolation method of choice is important for further analysis

of the vesicles, as they all have potentials and limitations, and thus needs to be carefully considered with the specific research question in mind.

1.6.5.2 Exosome visualization and characterization

Several methods can be applied for exosomal visualization. One of them is transmission electron microscopy (TEM). By using TEM, exosomes were first described to have a cup shaped morphology (128). However, this appearance was likely caused by sample preparation since later on using cryo-EM, for preserved exosomal shape, exosomes were described to have a round appearance (130). Of note, ultracentrifugation may affect the size and appearance of exosomes, due to vesicle collapse or fusion when high force is applied. Highly specialized flow cytometers can visualize antibody targeted exosomal surface proteins directly although often they lack the resolution required. Consequently, currently the most common way to characterize exosomes by flow cytometry, is based on beads coated with antibodies to capture exosomes. However, antibody-based capturing will select a subpopulation of vesicles positive for the particular marker for phenotyping, which can be avoided by the use of uncoated beads. A commonly used method to estimate the size distribution and concentration of vesicles is nanoparticle tracking analysis (NTA) (131), although it has been questioned whether NTA is robust enough to determine the vesicle concentration and give reproducible results. Importantly, NTA works better for mono-dispersed rather than poly-dispersed samples (132, 133). Other commonly applied methods for exosome phenotyping are ELISA for surface markers and western blot for proteins enriched inside of exosomes, such as Alix, Tsg101 and Hsp70 (117). The RNA profile of exosomes can be studied by the use of the chip-based capillary electrophoresis (Bioanalyzer), and individual RNAs can be analyzed by RNA sequencing, RNA microarrays and polymerase chain reaction (PCR). The exosomal RNA profile is dissimilar to cellular RNA and also the RNA profiles might differ depending on the vesicle type (121, 134). Moreover, the exosomal RNA isolation method of choice has been shown to influence the yield and quality of the extracted RNA (135). Taken together, applying several methods is recommended for exosome characterization however the sample amounts available often restricts the use of multiple methods.

1.6.6 Exosomes in cell-to-cell communication

Exosomes are intercellular messengers able to transport information over long distances to be received by distant cells (136). There are both specific and non-specific ways of exosomal binding and uptake by cells. The non-specific, clathrin-independent, uptake is achieved by macropinocytosis, phagocytosis or membrane fusion. Specific exosomal interaction with the plasma membrane surface receptors may induce intracellular signaling or clathrin-mediated, i.e. receptor-mediated endocytosis, binding and internalization via integrins (137), tetraspanins (138) and proteoglycans (139). The target specificity is controlled by the surface markers expressed on the exosome and the recipient cell (85, 116). Many examples of exosome and cell interactions can be found in the literature. For example, DC-derived exosomes carry the intercellular adhesion molecule (ICAM)-1 which is important for the interaction and activation

of T cells via integrin leukocyte function-associated antigen (LFA)-1 mediated exosomal capture (140). Moreover, LFA-1 expression on activated $CD4^+$ T cells facilitate the recruitment of MHC class II positive DC-derived exosomes (141). Exosomes can be transported through the blood stream and captured by marginal zone phagocytes in the spleen, by Kupffer cells in the liver or by macrophages in the lungs (31). CD169 is an important molecule for exosomal capturing in spleen and lymph nodes in mice, and induction of antigen-specific immune responses towards the exosomal carried antigens (142). $CD169^+$ macrophages in the lymph nodes act as tumor suppressors by capturing TEX and inhibit exosome promoted tumor progression (143). Organ selectivity has been further confirmed for cancer-derived exosomes, where target specificity to certain organs have been observed *in vivo*, termed organotropism, which is related to specific integrins present on the exosomes (144). Cancer-derived exosomes are also dependent on heparin sulfate proteoglycans for their uptake and function (139). A previous investigation of the exosomal fate *in vivo* presented that DC- and B cell-derived exosomes were complement resistant, which would suggest that they can provide a long-term effect (145). Recent studies propose a shorter exosome half-life in circulation although exosomes were still detectable in the spleen after being captured by MMM in the marginal zone (138, 142, 146). Furthermore, upon uptake the information carried by the exosomes, primarily proteins and RNA, are further processed by the recipient cell (147). In addition to delivering antigens to recipient cells, exosomes carry multiple signals that promote activation of host cells to provide an adjuvant effect (31). For example, glioblastoma-derived exosomes carrying functional mRNA have been shown to promote tumor growth *in vitro* (125). Exosomes may also deliver functional miRNA to recipient cells *in vitro* (147, 148), demonstrating that they can transfer information and be explored for therapeutic delivery of material. Moreover, exosomes can be loaded for transport of small interfering (siRNA), which can be delivered to tumor cells in order to knock down genes *in vitro* and *in vivo*. Also, exosomes loaded with mRNA have been successively transported to tumor cells. This suggests that exosomes plausibly can be used in RNA-based gene therapy in the future (149).

1.7 Exosomes and the immune system

1.7.1 Immune-stimulatory function of exosomes

The first study identifying a connection between exosomes and the immune system was the observation that B cell-derived exosomes carried MHC class II molecules that could present peptides to $CD4^+$ T cells and induce an antigen-specific response *in vitro* (94, 150). This motivated further investigations of exosomes and their role in immune stimulation. Exosomes originating from APCs carried MHC class I and class II molecules and were therefore potential inducers of $CD8^+$ and $CD4^+$ T cell responses, respectively (151, 152). For example, DEX presented immune-stimulatory properties by the activation of $CD4^+$ T cells *in vitro* (96), and successfully induced tumor-specific $CD8^+$ T cell responses *in vivo* (31). It has been shown that when exosomes interacted directly with T cells they induced a low immune-stimulatory effect, whereas when incubated with DCs they prompted efficient T cell stimulation (153). Thus, activation of DCs to prime T cell responses have been suggested as the major

mechanism *in vivo* (154). In contrast, direct T cell stimulation by exosomes was mainly observed when exosomes encountered $CD4^+$ T cells *in vitro* (155). Importantly, DCs were shown crucial for inducing immune activation in response to exosomes *in vivo* (156). Moreover, DEX efficiently transfer MHC class I-peptide complexes to DCs for the priming of $CD8^+$ T cell responses *in vitro* and *in vivo* (157). For immune activation, exosomes may be engulfed by DCs and the exosomal MHC/peptide complexes transferred "cross-dressed" to the DC surface, another possibility would be that exosomes stay attached to the DC surface to interact with the T cell directly (31, 158). MHC class II deficient DCs were able to stimulate antigen-specific $CD4^+$ T cell *in vivo*, suggesting the transfer of MHC complexes from exosomes to the DC surface for immune activation (152). After exosome internalization, the exosomal MHC/peptide complex can be recycled (151), or degraded by the DC and the peptides can be recycled (159). In addition, antigen-loaded exosomes need B cells to generate $CD4^+$ and $CD8^+$ T cell responses *in vivo* (160). Moreover, an immune-stimulatory effect by macrophage-derived exosomes have been observed *in vivo*, they specifically target the lymph nodes where they promote a pro-inflammatory cytokine environment (161). Studies have also demonstrated that tumor-derived exosomes (TEX) can activate immune cells *in vitro* by carrying MHC class I molecules and heat shock proteins, and that they may also provide an anti-tumor effect *in vivo* (162). In support of this, TEX loaded DCs injected in tumor bearing mice induced more antigen-specific $CD4^+$ T cells, stronger anti-tumor response and prolonged survival compared to tumor-lysate loaded exosomes (163).

1.7.2 Immune inhibitory function of exosomes

Exosomal function is dependent on their cellular origin, thus many exosomes have shown an immune inhibitory capacity, e.g. tumor-, placenta-, gut- and stem cell-derived exosomes. Notably, the route of administration in different experimental models also affects whether exosomes will induce an activating or inhibitory immune response. For example, immune suppressive functions have been described for intestinal epithelial cell line-derived exosomes "tolerosomes" exposed to OVA and from serum-derived exosomes in mice after oral antigen administration (164). Upon oral administration of OVA, tolerosomes were able to prevent allergic sensitization in a mouse model for asthma and these mice presented higher levels of Tregs and lower levels of IgE (165). Also, bronchoalveolar lavage fluid (BALf)-derived exosomes from mice exposed to the pollen allergen (Ole e 1) showed immune suppressive functions such as the inhibition of IgE responses and increased TGF-β production and prevented allergic sensitization in the recipient mice (166). Plasma-derived exosomes from mice enriched in MHC class II molecules and FasL displayed an antigen-dependent immune suppressive function (149). Furthermore, placenta-derived exosomes have an immune suppressive role during pregnancy (167), partly mediated through the expression of FasL and TRAIL on the exosomes important for induction of apoptosis of activated immune cells for maintaining immune suppression (168). Interestingly, placenta-derived exosomes have been shown elevated in plasma of mothers that deliver at full term, as compared to those that deliver pre-term indicating a pregnancy protecting effect of these exosomes (169). Moreover, tumor cell line secreted exosomes, may also induce immune suppression and contribute to

tumor progression and metastasis (31, 170). Tumor exosomes carry tumor-associated antigens and immune inhibitory markers, such as FasL, TRAIL and PD-L1 (171). Notably, it has been shown in co-culture experiments that TEX suppress the effector $CD8^+$ T cells (172, 173), and promote expansion of Tregs, further supporting an immune inhibitory environment (174). Not only T cells are inhibited by TEX co-culturing, also other immune cells, e.g. monocytes, B cells, NK cells and DCs respond to the immune suppressive signals expressed by TEX. For example, TEX prevented monocyte differentiation and induced TGF-β expression by DCs for further immune suppression (171). Notably, TEX derived from metastatic cell lines promote more metastasis compared to exosomes from non-metastatic cell lines, and they also stimulate a pro-metastatic phenotype of bone marrow progenitors (170). In addition, injection of the tumor cell line E.G7-derived exosomes expressing OVA was non-immunogenic *in vivo* (156), further supporting an immune evading capacity of tumor exosomes.

1.8 Exosomes in cancer immunotherapy

Since exosomes were first shown to carry functional MHC/peptide complexes, the use of DEX has evolved as a promising approach in immunotherapy. The first attempt to use a cell-free approach in cancer treatment was successfully performed by the use of DEX in a mouse melanoma model (96). This led to the start of two phase I clinical trials using DEX in melanoma (175) and in non-small cell lung cancer (176). The main conclusions from these two clinical studies were that it was possible with large scale production of DEX and that they were safe to be administered in humans. However, these studies failed to show a sufficient upregulation of CTLs in the patients, which probably depended on the late stage tumor status and also the use of immature DCs for exosome collection. In a recent phase II clinical trial, using IFN-γ matured DEX loaded with MHC class I and class II tumor peptides, DEX were shown able to induce NK cells while negligible T cell responses were observed in patients with lung cancer (177). DEX expressing NKG2D ligands have been shown to activate NK cells by binding NKG2D *ex vivo* and *in vivo*, and also to improve metastatic control via $NK1.1^+$ cells (178). Notably, the addition of LPS or IFN-γ to the DC culture before exosomal isolation improved their immunogenic capacity by upregulation of exosomal costimulatory markers (140, 150). The use of peptide-loaded exosomes was not sufficient to trigger strong anti-tumor immune responses in the human clinical setting and another limitation was the use of an autologous system. It has been suggested that an allogenic system would serve as an alternative without causing severe side effects. Thus, allogeneic exosomes have been shown to deliver antigens, stimulate immune cells and suppress tumor growth and are therefore suggested as a potent cell-free cancer vaccine (179). Importantly, not only DCs are important for a good exosome-based vaccine response, also B cells are crucial for mounting $CD4^+$ and $CD8^+$ T cell responses as previously demonstrated (160, 180). This propose the use of whole antigens on exosomes for therapy and not solely loading of T cell peptides for immune activation. Further suggesting that allogeneic exosomes may be used, since the direct exosome and T cell receptor interactions via MHC/peptide complexes are negligible *in vivo*.

Contradictive data propose a dual function of TEX, as both inhibitory and stimulatory functions have been described. Their role in the immune therapeutic setting was suggested to be defined by the TME and the cells targeted by the TEX treatment (171). Importantly, TEX naturally transport tumor antigens that can be taken up by DCs and be further presented to CTLs to induce an anti-tumor specific immune response. Interestingly, both syngeneic and allogeneic TEX have been confirmed equally potent in tumor eradication (161). The first phase I clinical trial combining tumor-derived exosomes with granulocyte-macrophage colony-stimulating factor (GM-CSF) was performed in patients with colon cancer, unfortunately tumor-specific $CD8^+$ T cell responses were only detected in some patients (181). Importantly, TEX has been shown less efficient in induction of $CD8^+$ T cell responses compared to DEX (156).

Other stimuli may be complemented to the exosomes to further potentiate their immune-stimulatory function. The addition of the NKT ligand α-galactosylceramide to the DEX enhanced the immune response by the engagement of invariant iNKT cells, which also inhibited tumor progression in mice (182). DEX co-administered with the TLR9 ligand CpG-ODN also successfully boosted the anti-tumor immune response *in vivo* (183).

Mesenchymal stem cell (MSC)-derived exosomes have mainly been tested in clinical trials for GvHD and chronic kidney disease due to their immune suppressive nature (184, 185). MSC-derived exosomes may plausibly also provide an anti-tumor therapeutic alternative. However, the role of MSC-derived exosomes in cancer treatment needs to be further explored (186). So far, the exosomal induced immune-stimulatory effect on cells and their ability to induce tumor regression observed by diverse sources of exosomes in mouse models have unfortunately not corresponded well with the observations made in clinical trials.

1.9 Exosomes in disease diagnostics

Exosomes in body fluids reflect their cell or tissue of origin and selective loading of proteins to exosomes suggests that they may serve as potential noninvasive biomarkers. Altered exosome quantities and their cargo have been associated with a wide range of diseases; sarcoidosis (187), cancer (100, 188), Alzheimer's disease (189), cardiac disease (190), and asthma (99, 191), and thus exosomes might serve as easily accessible "liquid biopsies" for biomarkers or prognostic markers of disease (162, 192, 193).

Several studies investigating the protein content of exosomes from diverse sources using different techniques have been performed and numerous proteins have been associated with cancer diagnosis and prognosis (98, 144, 194-196). For example, melanoma-derived exosomes present a "melanoma profile", by the transport of proteins, such as MET, TYRP2 and Hsp70 important for metastasis formation, demonstrating their potential as disease markers (170). In addition, serum-derived vesicles from glioblastoma patients contain epidermal growth factor receptor (EGFR) to a higher extent compared to controls, thus potentially indicating their diagnostic capability (125). From a biomarker perspective, finding highly specific early detectable markers are crucial. One such protein is the glypican-1 identified as an early

pancreatic cancer marker in human plasma-derived exosomes, which also correlated closely to tumor burden and could furthermore be linked to prognosis (197). Notably, proteomic approaches to find exosome biomarkers have been extensively investigated (116, 198, 199). Taken together, they all suggest that exosomal protein content can be investigated in a biomarker perspective. In contrast, the lipid profile of exosomes has recently become more explored using sensitive mass-spectrometry (200, 201).

Exosomes have also been shown to transport tumor-derived mRNA and miRNA, which also increased their interest from a biomarker perspective. Since then, the exosomal biomarker field has largely focused the attention on exploring the correlation of exosomal RNA and disease diagnosis (170, 202). For example, the levels of selected miRNAs observed in ovarian cancer-derived exosomes correlated to the miRNA profile of the tumor cells. Importantly, these miRNAs were uniquely expressed in the TEX and absent in healthy ovarian tissue suggesting their potential as biomarkers (203). In another study, serum-derived exosomes from glioblastoma patients presented an overall increase in total RNA content in comparison to healthy individuals and a reduction in some specific rRNAs which are objects for further investigations as biomarkers for glioblastoma (204).

In conclusion, sensitive methods feasible for high-throughput analyses are necessary for exosomal investigation of disease diagnostic and prognostic markers. New screening methods are under development and collection of data in comprehensive data bases will further contribute to the potential to reveal disease patterns for predicting prognosis. At present, several clinical trials investigating exosomes as biomarkers for diverse cancers are ongoing and the outcomes of these trials remain to be revealed. Hopefully, improved disease diagnostics will be available in the near future, as the screening possibilities will also provide more individualized treatment strategies.

2 THESIS AIMS

The main focus of this thesis was to study exosome-induced immune responses *in vivo* and how to improve their stimulatory capacity for immunotherapy. In addition, this thesis explored a novel technology for identifying protein profiles in human body fluid-derived exosomes for improving the applicability of exosomes for diagnostics purposes.

Study I

This study aimed to investigate the role of MHC class I molecules on exosomes to identify whether MHC/peptide complexes are required on the surface of exosomes to trigger antigen-specific $CD8^+$ T cell immune activation *in vivo* and to explore the application of allogeneic exosomes in cancer immunotherapy.

Study II

The aim of this study was to, as a follow-up to study I, evaluate the ability of allogeneic exosomes to activate an antigen-specific immune response and examine the effect of repeated injections of allogeneic exosomes on the development of immune memory and to further explore their immune therapeutic potential in a tumor model.

Study III

The aim was to explore the use of lyophilization as a novel strategy for antigen and adjuvant loading of macrophage cell line RAW 264.7-derived exosomes and to test their immune-stimulatory potential and their capacity to reduce tumor progression *in vivo*.

Study IV

This study aimed to examine the proximity extension assay (PEA) as a means of identifying exosomal proteins in small sample volumes and to distinguish differential protein patterns between cell lines and their corresponding exosomes, with the purpose to investigate the feasibility of using PEA as a diagnostic tool.

3 METHODOLOGY

All methods used in this thesis are described in detail in the individual studies. Thus, this part serves to provide a broader overview of the methods applied and also to identify methodological considerations in the exosome field and the studies performed. Although, there are limitations to the individual methods, they serve as a complement to each other and contributes to the final understanding and interpretation of the results.

3.1 Mice

All animal experiments were approved by the local ethics committees. In study I-III, C57Bl/6 and BALB/c mice were used. MHC class $I^{-/-}$ mice (lacking both H2Kb and H2Db genes) were kindly donated by Klas Kärre (Karolinska Institutet) and OT-I/$Rag2^{-/-}$ mice with TCR specific for $OVA_{SIINFEKL}$ peptides, both on a C57Bl/6 background, respectively were bred at Karolinska Institutet, MTC animal facility. The experiments are explained in detail in the individual studies performed and summarized in figure 4. In brief, to examine immune stimulation *in vivo*, exosomes were injected intravenously (i.v.) (study I-II) or intraperitoneally (i.p.) (study III). Notably, the injection routes are suggested to affect the fate of the exosomes, and exosomes injected i.v. are systemically distributed and predominantly target the liver, as compared to i.p. injected exosomes, which largely interact locally with tissue resident cells. Consequently, the injection route of choice should be considered as it will plausibly affect the outcome of the study. Therefore, additional studies where injection routes are compared side by side are desired. Not only the injection route will affect the distribution, also exosomes of different sources carry adhesion molecules that will direct them to certain target cells and organs.

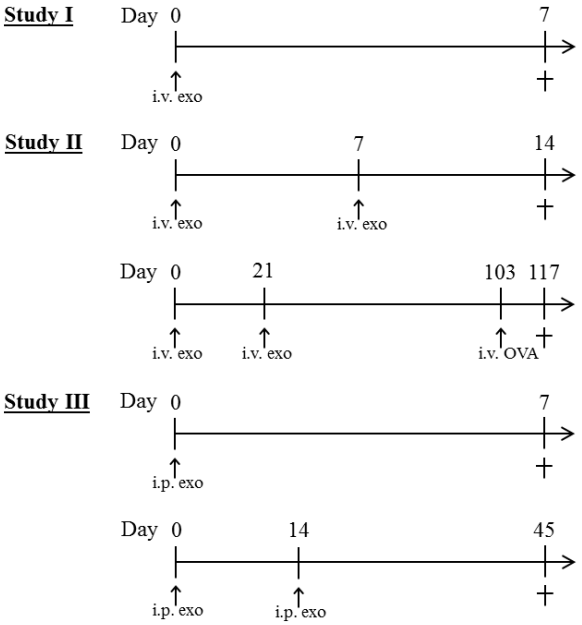

Figure 4. Schematic overview of the exosome immunization schedules in the different studies I-III.

3.2 Tumor models

In the B16-OVA F1 melanoma model (study I), 30,000 or 200,000 tumor cells were injected subcutaneously (s.c.) in female mice for tumor establishment. For the B16m-OVA F1 melanoma model (study II), 50,000 tumor cells were injected s.c. in female mice and tumor development was confirmed. In both study I and II, bone marrow-derived dendritic cells (BMDC)-derived exosomes were injected i.v. as a therapeutic treatment model to prolong survival, and the tumor growth was followed until a tumor size of 1000 mm^3 was reached. In the B16-OVA F10 melanoma model (study III), female mice were injected s.c. with $1x10^6$ tumor cells. This model was used as an immunotherapeutic approach and exosomes were injected in mice with established tumors. In the E.G7-OVA thymoma model (study III), male mice were injected s.c. with $5x10^6$ tumor cells. This model was used to challenge mice that had been pre-immunized with exosomes to evaluate if they can be used as a protective cancer vaccine. In study III, the tumor growth was followed and the experiments were terminated day 17 or day 21 for the B16-OVA and at a tumor size of 4000 mm^3 for the E.G7-OVA (figure 5). Of note, in study III, RAW 264.7-derived exosomes were injected i.p. in both the B16-OVA and the E.G7-OVA model. Taken together, all tumor models were used with an explorative purpose to find therapeutic strategies using exosomes which can also be applied on other tumor types.

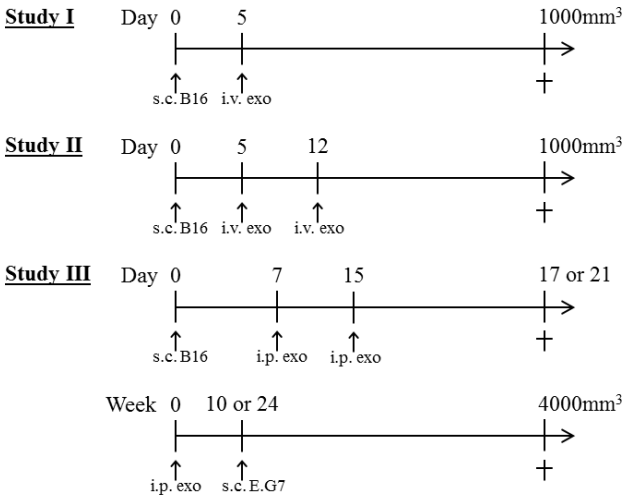

Figure 5. Schematic overview of the exosome immunization schedules in the tumor models in the different studies I-III.

3.3 Primary bone marrow-derived dendritic cell cultures

For the generation of exosomes (study I and II), primary BMDCs were cultured from C57Bl/6, MHC class $I^{-/-}$ or BALB/c mice, respectively. The culture medium was supplemented with recombinant IL-4 and GM-CSF containing culture medium generated from A8653/X63 clone, a kind gift from Mattias Svensson (Karolinska Institutet), for stimulating DC differentiation. On day 6, 300 μg/ml OVA and, when required, 100 ng/ml αGC was additionally added to the cultures followed by an overnight incubation. On day 7, cell culture medium containing exosome-depleted fetal calf serum (FCS) was supplemented with LPS for DC maturation, as

LPS is essential for BMDC secretion of immune-stimulatory exosomes. After 48 h in culture, the supernatant was collected for exosome isolation.

3.4 Cell cultures

The murine macrophage cell line RAW 264.7 was used to generate cell culture supernatant containing exosomes (study III). This cell line was selected as their exosomes have been demonstrated less immunogenic compared to DC-derived or tumor cell-derived exosomes. Therefore, their function as delivery tools of OVA, CpG-ODN and αGC could be solely studied without interference of an immune stimulation generated by the vesicles themselves. In study IV, various cell lines were used for the assessment of the PEA on protein content of cells and their corresponding exosomes. All cell lines used were cultured according to the supplier's instructions.

3.5 Healthy human subjects

In study IV, sample collection was performed from healthy volunteers. Breast milk from non-allergic mothers was collected at Karolinska Institutet and processed directly or stored at 4°C and handled within 12 hours of sampling. Seminal fluid was collected at Uppsala University Hospital. The study was approved by local ethics committees and informed consent was obtained from all donors.

3.6 Exosome isolation

Throughout the studies presented in this thesis differential centrifugation was applied for exosome isolation. Depending on the exosome source such as cell culture supernatant or human body fluid the protocols were slightly modified for optimization of exosomal purity and yield. In some protocols, additional purification using sucrose gradient was applied (study IV). In brief, when working with human body fluids (study IV), these samples contain immune cells, proteins and lipids. For a successful exosome isolation, additional sample dilutions prior to vesicle isolation were required, followed by inclusion of further washing steps in order to remove contaminations from the vesicle preparations. In study IV, density gradient purification was not applied on the milk exosome preparations as this would potentially reduce the sample yield. However, this additional purification step will be considered in future studies when milk exosomes are used. For isolation of prostate-derived exosomes "prostasomes", the sample purification protocol had been carefully optimized previously and included sucrose gradient purification, as described in study IV. For cell line-derived exosomes, the isolation protocols found in the literature might slightly differ. In the present study, all cell line-derived exosomes were isolated in a standardized way. Cell culture supernatants are less likely to have contaminating proteins, other than the FCS supplemented to the culture medium, which were excluded before final incubation and exosome isolation. Therefore, additional washing steps were not considered to be required. In brief, common for all exosome isolation protocols used here are the initiation by removal of cells and cell debris. When applicable, samples were filtered through a particle size restricted filter to remove larger vesicles, followed by

ultracentrifugation for exosome isolation and subsequently samples were stored at -80°C until further use. Ultracentrifugation has been questioned due to the unselective isolation and co-pelleting of contaminants as the force applied would concentrate all particles heavy enough/of a particular size and weight. Moreover, there is also a risk for vesicle fusion due to the centrifugation force. Despite the limitations, ultracentrifugation is still considered as the "golden standard" technique for exosome isolation. Another limiting factor in the exosome field is the frequently low vesicle yield after isolation which will impact downstream applications such as characterization and functional studies *in vitro* and *in vivo*. Consequently, method optimization and controls used in the experiments always have to be carefully selected.

3.7 Flow cytometry

Flow cytometry was used for defining cell populations based on their surface marker expression. In brief, the initial incubation with Fc-block to avoid unspecific antibody binding was followed by addition of fluorophore conjugated antibodies. The cells were investigated using a flow cytometer for single cell identification of live cells, and the diverse cell populations were defined in the different studies (study I-III). Additional fixation and permeabilization steps followed by antibody incubation was applied to assess *in vivo* proliferation of cells using bromdeoxiuridin (BrdU) incorporation. In addition, intracellular production of IFN-γ (study I and III) was explored by incubating the cells for 4 hours *ex vivo* using PMA, Ionomycin and Brefeldin A to activate the cells, stimulate cytokine production and block intracellular protein transport, respectively. In addition, exosome surface markers were analyzed by flow cytometry. However, due to their small size of 30-150 nm in diameter, directly distinguishing true signals from the background is challenging using available flow cytometers. Instead, antibody conjugated sulfate-aldehyde latex beads were used for exosome binding to enable visualization of exosomal surface markers (figure 6). The purified antibody of choice varies depending on the exosome source to be investigated and needs to be defined for each vesicle population. Here, anti-CD9 conjugated beads were used for BMDC-derived exosomes (study I and II) and anti-CD81 conjugated beads for the RAW 264.7-derived exosomes (study III) for exosome characterization. Of note, the use of antibodies for the bead-based approach, thus select for the vesicle population comprising the marker of choice.

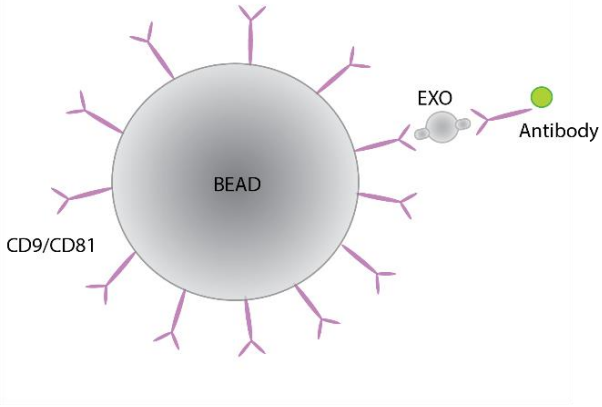

Figure 6. Bead-based capture of exosomes for phenotyping using flow cytometry. Latex beads were coated with an antibody (anti-CD9 or anti-CD81) for exosome capture, followed by staining with fluorophore conjugated antibodies for identification of exosomal surface marker expression.

3.8 Western blot

Exosomal proteins are both surface exposed and found inside the vesicles. For investigation of intravesicular proteins, flow cytometry is not feasible since the antibodies are not able to penetrate the lipid membrane of the vesicles. Gentle fixation protocols used for intracellular markers are not capable of penetrating the rigid exosomal lipid membrane. Instead, exosomes are lysed using strong detergents such as RIPA buffer, for extraction of vesicle proteins followed by repeated vortexing and sonication for western blot identification of intravesicular proteins. In all studies I-IV, western blot was applied for the purpose of vesicle profiling and additionally to determine the amount of OVA protein carried by the vesicles (study I-III). Unfortunately, for exosomal protein evaluation using western blot, there are no housekeeping genes able to serve as internal loading controls. Here, the Turbo blot system and their pre-casted gels is of advantage, as UV light can be applied directly on the gel for visualization of total protein amount loaded and thus serves as a control for total protein loading onto the gel.

3.9 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a quick and specific antibody-based method to analyze and quantify proteins, e.g. secreted cytokines and antibodies in cell supernatant or serum. ELISA was used to estimate OVA levels on exosomes to ensure that the injected exosomal batches had similar levels of surface antigen (study I-III). In addition, ELISA was also used to examine antibody subclasses in serum of mice after exosome immunizations (study I-III). No blocking was included as the mouse serum contain bovine serum albumin antibodies induced by the exosomal carried FCS originating from the cell culture medium, which would generate a false positive signal. In study III, the pro-inflammatory cytokines (IL-6 and IL-12) released to the splenocyte cell culture medium *in vitro* were assessed and quantified. ELISA provides an easy platform for the investigation of cytokines in serum or cell culture medium, with the limitation that the cytokine production is transient and the kinetics require time point optimization for the different cytokines for assessment of cytokine expression after *in vitro* stimulation.

3.10 Enzyme-linked immunospot (ELISpot) assay

ELISpot, a sensitive method for quantification of cells releasing cytokines, was used for examining the IFN-γ release by splenocytes upon *ex vivo* restimulation (study I-III). Splenocytes were added to the IFN-γ capture antibody coated plates for overnight culture together with different stimuli; peptides for T cell stimulation (CD4 peptide: $OVA_{323-339}$ and CD8 peptide: $OVA_{SIINFEKL}$), whole OVA protein for B cell stimulation or αGC for iNKT cell activation (study I-III). In addition, ELISpot was used for detecting antibody producing cells (study II) by coating the plates with anti-mouse IgG and adding splenocytes for plasma cell identification followed by using biotinylated OVA for detection of the antigen-specific antibody producing cells. This was followed by visualization and quantification of spot forming units (SFU)/10^6 cells using the iSpot FluoroSpot Reader System. The advantage with ELISpot, compared to cell supernatant analysis using ELISA, is that all events that occurs during the incubation time will be captured, which reduces the need for optimization dependent on

cytokine kinetics, and thus ELISpot serves as a complement to ELISA. Furthermore, the high sensitivity is an advantage for cytokines that are only released in small amounts. Importantly, cells will be evaluated for their secretion capacity in response to a specific stimuli. In contrast to the assessment of intracellular cytokine production using flow cytometry (previously described), where unspecific stimulation and blocking cytokine release affect the health status of the cells. Notably, ELISpot do require; i) optimization of cell numbers used in each well for the stimulation, and ii) adaptations of the settings for defining positive signals, SFU, using the plate reader. In addition, compared to intracellular cytokine staining using flow cytometry, ELISpot is not able to identify the cell that produced the cytokine if a mixed cell culture is used. For multiple cytokine analysis FluoroSpot can be applied according to the same principles as ELISpot, to provide information about cytokine release from a certain cell, compared to other multiplex assays for example Luminex which would only provide concentrations of the cytokines in the samples, but not reveal which cells they were secreted from, nor if the same cell secreted the cytokines.

3.11 Electron microscopy

As exosomes are nano-sized particles, high resolution microscopy is needed for visualization. Here, BMDC-derived exosomes from C57Bl/6 and BALB/c mice were identified by transmission electron microscopy (TEM) with a negative ion capture, staining the background and leaving the sample unaffected but visible (study II). There are different techniques available, e.g. TEM, scanning electron microscopy (SEM) and cryo-EM. TEM provides higher resolution than SEM. TEM and cryo-EM are similar with the difference that cryo-EM is performed at low temperatures using liquid-nitrogen, which is important for temperature sensitive samples and leaves the vesicles more intact, i.e. spherical. A small sample volume was added to a grid and stained with 5 µl 1% uranyl acetate before visualization in a Hitachi HT 7700 electron microscope. Images were obtained using a Veleta camera (Olympus).

3.12 Lyophilization

Study III comprises a novel method for external exosome loading using lyophilization. In study I and II indirect loading was applied to introduce antigen to the exosomes. However, indirect loading is not applicable on all cell lines as they might lack receptors for antigen uptake and consequently are unable to take up and process the antigen. As the RAW 264.7 cell line was not taking up OVA directly from the culture medium, the lyophilization technique (a freeze dry method) was applied (study III). Antigens and adjuvants were added to the exosome solution in phosphate-buffered saline (PBS) and they were snap-frozen followed by overnight lyophilization. The dried pellet was reconstituted with water and PBS to provide the encapsulation of antigens to the exosomes. Vesicle characterization was extensively compared before and after lyophilization and revealed the recovery of exosomal features. Importantly, the antigens and adjuvants were found co-localized with the vesicles. This method solves several problems; i) facilitates loading of antigens directly to exosomes, ii) allows loading of antigens that are not taken up by cells, iii) enables loading of innate stimuli such as CpG-ODN to exosomes without affecting the health status of the cells.

3.13 Bioanalyzer

Exosomal RNA was isolated using the miRCURY RNA isolation kit. The total RNA profiles, yield and size distribution, of RAW 264.7-derived exosomes before and after lyophilization were assessed in study III using the chip-based capillary electrophoresis method termed Bioanalyzer. The RNA 6000 Pico kit for total RNA was used according to manufacturer's instructions. Exosomes mainly contain small RNA, which gives them a different RNA profile compared to cells.

3.14 *In vitro* proliferation

OT-I/Rag2$^{-/-}$ mice with T cell receptors specific for the $OVA_{SIINFEKL}$ peptide presented by the MHC class I molecules were used to study antigen-specific T cell proliferation in a splenocyte culture *in vitro* in response to OVA loaded exosomes presenting the CD8 peptide SIINFEKL on their MHC class I (study I and III). Cells were stained with the stable fluorescent staining carboxyfluorescein succinimidyl ester (CFSE) that will penetrate the cell membrane. Proliferation can be followed as the staining intensity will gradually decrease for each cell division. The cells were incubated for 5 days to quantify proliferation of antigen-specific T cells *in vitro*.

3.15 Size distribution analysis of exosomes

3.15.1 Nanoparticle tracking analysis

To investigate the size distribution of BMDC-derived exosomes, the nanoparticle tracking analysis NTA was used (study I and II). Vesicle size was determined based on the rate of light scattering and the Brownian motion of the particles. This technique works well for mono-dispersed compared to poly-dispersed samples, as the threshold would select the vesicle size to be identified and the mean vesicle size will be presented, thus making the method biased if vesicles of different sizes occur. The number of particles (concentration) can also be calculated using NTA however of different accuracy depending on the calibration of the machine and therefore might not be fully reliable.

3.15.2 DLS analysis and AFM topography

In study III, RAW 264.7-derived exosomal size distribution was evaluated using the Zetasizer dynamic light scattering (DLS). In DLS the laser light hits the vesicles and scatters in all directions. This method is limited to small particles in suspension at a low particle number and is therefore suitable for exosome research. DLS is a similar technique as the NTA although without the ability to calculate the particle concentration. In addition, the vesicle size distribution was further assessed by the use of atomic force microscopy (AFM) (study III). The high resolution microscopy will scan the surface of the dried sample by using a mechanical probe to create an image of the sample structure. The purpose of using DLS and AFM was to investigate the exosome characteristics before and after lyophilization. Of note, particle sizes are affecting their uptake by cells *in vitro* and *in vivo*.

3.16 Exosome staining for uptake studies

Exosomes were stained using the PKH67 lipophilic membrane dye for exosome cell interaction/uptake evaluation *in vivo* (study II), as a control free dye was injected. 1h after i.v. injection no signal from the exosomes were detected *in vivo* and only MHC class II upregulation was observed on splenic DCs, monocytes and macrophages. Moreover, in study III exosomes were stained with the lipophilic dye SP-DiOC, which is highly fluorescent when fused with membranes and with negligible fluorescence in water, thus providing less background than other comparable lipid-based membrane dyes. After staining the exosomes, they were incubated with RAW 264.7 cells for *in vitro* uptake evaluation (study III). Different time points and also active uptake (37°C) and passive uptake (4°C) was assessed by flow cytometry and confocal microscopy.

3.17 Proteomics

The PEA technique (Olink Proteomics) was applied to investigate the feasibility to study exosome protein content (study IV) (figure 7). The advantage of PEA over other commonly used proteomic methods is the small sample volume required as a result of the increased sensitivity due to the PCR amplification, which is an advantage in the exosome field as sample amounts are often the limiting factor. The PEA method applies two DNA-conjugated antibodies targeting different epitopes of the same protein, which brings the DNA arms in close proximity and facilitate the hybridization of the DNA arms to each other, followed by enzymatic DNA polymerization to form a new double-stranded DNA molecule that is used as template for signal amplification by PCR (figure 7). Thanks to the dual target recognition, the reported cross-reactivity and non-specific signals are minimized. Several antibody panels were applied, those already available for cancer and cardiovascular diseases and those, at that time, under development (cell cycle, cancer and neuro-oncology), comprising 92 proteins and internal controls each. Several cell lines, and their corresponding purified exosomes were assessed to evaluate the performance of the multiplex PEA for proteome analysis of exosomes (study IV). Also, human body fluid-derived exosomes from healthy controls were evaluated for the feasibility to apply PEA on body fluid vesicles. The limitations of this technique are; i) similarly to other affinity-based technologies, antibody binding properties varies, not allowing direct comparison of protein concentrations, ii) The current protein panels are fixed, not allowing custom designing of panels, and iii) the platform allows relative quantification rather than absolute quantification of each protein. On the other hand, from a diagnostic and biomarker perspective when the protein of interest is already known, this method could serve as a possible tool to assess clinical samples due to the small sample size required.

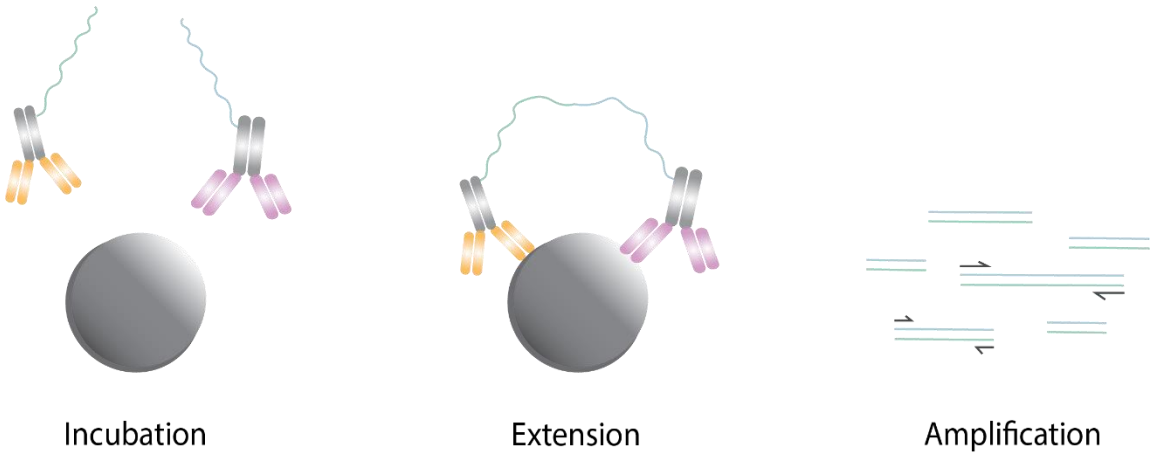

Figure 7. Principle behind the PEA technique, two antibodies recognizing different epitopes on the same protein are incubated with the sample. Matched antibody pairs linked to unique DNA sequences will hybridize, when binding in close proximity this will induce extension by DNA polymerase and amplify the positive signal by PCR.

3.18 Data analysis

After generating the proteomic data using the PEA method (study IV) the limit of detection (LOD) and normalized protein expression values (NPX, expressed in log2-scale) were calculated. The values were then applied for data analysis using R (RStudio) after correction for background noise. In brief, a 2-fold cut-off of the LOD standard deviation (corresponding to a 95% confidence interval) was subtracted from the NPX values. The final corrected NPX values were used to generate the heatmaps, perform principal component analysis (PCA) and hierarchical clustering presented in study IV. The ComBat analysis removed specific protein signals present solely in cell lysates or exosomes to enable tracing of exosomes to its originating cell line by only comparing the protein similarities. Pearson correlation was used in combination with a hierarchical clustering algorithm to compare sample distances. The bootstrapping algorithm (pvclust package) was used to estimate statistical certainty of the hierarchical clustering. To allow the comparison between milk- and prostate-derived exosomes, the data distribution was first corrected using quantile normalization. Sample comparison was performed using empirical Bayes statistics to assess the differentially expressed proteins.

3.19 Statistical analyses

The statistical analyses are explained in the individual studies. In brief, all analyses were performed using GraphPad Prism 6.0 and presented as mean ± SEM (unless otherwise stated) and considered significant if $p<0.05$ (Study I-III). Student's t-test or one-way ANNOVA with Bonferroni's correction were used for normally distributed data. Mann-Whitney or Kruskal-Wallis test with Dunn's correction were applied for non-parametric data. For the tumor models, survival was evaluated using Kaplan-Meier survival curve and the data were analyzed using Mantel-Cox test.

4 RESULTS AND DISCUSSION

This part serves to provide a short summary of the findings in the different projects, as they are discussed more detailed in the respective studies included in this thesis. In addition, a combined reflection on the overall findings can be found at the end of this section.

4.1 Main findings

4.1.1 Study I

In this study, we found that BMDC-derived exosomes that carry the whole OVA antigen are able to stimulate antigen-specific $CD8^+$ T cells both *in vitro* and *in vivo* even in the absence of exosomal MHC class I expression. The immune boosting effect related to engagement of iNKT cells achieved by the additional use of αGC on exosomes was further investigated in the context of exosomes lacking MHC class I. We could clearly detect the additional effect of αGC on exosomes in enhancing the immune response by boosting iNKT cell activity. In addition, splenocytes that previously had been exposed to wildtype or MHC class I lacking ($MHCI^{-/-}$) exosomes were restimulated *ex vivo* using the MHC class I peptide $OVA_{SIINFEKL}$ or whole OVA protein. Both groups were shown to induce IFN-γ production in similar levels, thus demonstrating equally potent T cell stimulation. Furthermore, we wanted to investigate the role of MHC molecules for their potential to reduce tumor progression in the B16-OVA melanoma model. For this purpose, wild type exosomes, $MHCI^{-/-}$ exosomes and MHC mismatched (allogeneic) exosomes were investigated for their tumor protective effects. All treatment groups had comparable levels of antigen-specific $CD8^+$ T cell infiltration into the tumor. They also produced similar amounts of antigen-specific antibodies (OVA IgG) in the serum and increased overall survival of the mice compared to the control. Taken together, this shows that immune responses to exosomes are independent of MHC/peptide complex presentation in the presence of the whole antigen, suggesting that a direct exosome MHC/peptide complex engagement of T cells is not the major event taking place *in vivo*. Instead, it indicates that the whole exosome carried antigen is processed by APCs, which then presents the peptides to T cells to mount an immune response. Importantly, this study demonstrates the ability to use impersonalized exosomes for immune therapy, which would serve as a time-saving and cost-beneficial therapeutic approach in the clinic.

4.1.2 Study II

In follow-up to study I, we here further investigated the immune responses induced by the allogeneic exosomes and also compared the immune-stimulatory effect after two injections. This study demonstrate that allogeneic exosomes are more efficient than syngeneic ones in boosting an immune response shortly after two i.v. injections. We observed an upregulation of Tfh cells and antigen-specific antibody production in response to allogeneic exosomes compared to the syngeneic ones when investigating the immune response 14 days after initial immunization. Regarding their ability to induce T cell activation, we observed that, syngeneic and allogeneic exosomes were equally efficient in mounting an antigen-specific $CD8^+$ T cell response *in vivo*. Moreover, splenocytes from both syngeneic and allogeneic exosome-injected

mice induced comparable amounts of IFN-γ upon restimulation with the MHC class I peptide $OVA_{SIINFEKL}$ *ex vivo*. Taken together, both exosomal sources demonstrated similar capabilities of T cell induction. In contrast, the allogeneic exosomes displayed an increased potential to induce B cell responses. Notably, a significant increase in IFN-γ production upon *ex vivo* restimulation of splenocytes in response to whole OVA was only observed by splenocytes that had encountered the allogeneic exosomes. In addition, allogeneic exosomes generated higher antigen-specific antibody titers compared to syngeneic exosomes after the initial injections although gradually decreasing superiority of allogeneic exosomes was observed over time. Importantly, the antibodies generated after two injections had higher avidity and more antigen-specific antibody producing cells i.e. plasma cells thus supporting multiple injections for induction of stronger immune responses when used in therapies. When mice were challenged with OVA protein four months after the initial exosome injections a reactivation of the immune response was observed. The antibody levels quickly increased, indicating the formation of an immune memory. However, we were not able to observe the presence of antigen-specific $CD8^+$ T cells or memory T cells ($CD4^+/CD8^+$), $CD62L^{low}$, $CD44^{high}$) in the spleen at the end of the long-term experiment. We also compared two injections of syngeneic or allogeneic exosomes in the B16m-OVA melanoma model. Our data demonstrate that both syngeneic and allogeneic exosomes induced similar levels of antigen-specific responses such as tumor infiltrating $CD8^+$ T cells, antibody production, significantly delayed tumor progression and enhanced survival time of the mice compared to the control. In conclusion, this study shows that allogeneic exosomes initially induce a stronger antigen-specific immune response and markedly improve the amount and avidity of the antigen-specific antibodies. Furthermore, our data provides additional knowledge of the effects of allogeneic exosomes *in vivo*, and suggests their potential use in impersonalized immune therapies in clinical studies.

4.1.3 Study III

In this study, we investigated lyophilization as a novel method for loading of RAW 264.7-derived exosomes with antigens and adjuvants to explore their immune-stimulatory capacity *in vivo*. Initially, a thorough comparison of the exosome characteristics before and after the lyophilization was performed to validate whether they retain exosomal properties i.e. size and surface marker expression. We showed that the expression levels of MHC class I and the tetraspanins; CD9, CD81 were preserved and at comparable levels after exosome reconstitution. This was followed by vesicle loading with whole OVA protein, the TLR9 ligand CpG-ODN and iNKT stimulating αGC, which was successfully performed and carefully evaluated. In order to explore whether these exosomes accomplish activation of immune cells, we started with investigating them *in vitro*. We clearly demonstrated that the OVA loaded RAW 264.7-derived exosomes induced $CD8^+$ T cell proliferation *in vitro* by using splenocytes from OT-I/$Rag2^{-/-}$ mice. Importantly, we also detected a stronger *in vitro* proliferation when exosomes were additionally loaded with CpG-ODN and αGC, suggesting that CpG-ODN further stimulate DCs for improved T cell proliferation *in vitro*. Thereafter, we investigated their immune-stimulatory effect *in vivo* and could confirm that loaded RAW 264.7-derived exosomes after i.p. injection induce an overall $CD4^+$ and $CD8^+$ T cell proliferation, upregulate

antigen-specific $CD8^+$ T cells and stimulate the production of antigen-specific antibodies in the serum. Moreover, we observed that tumor progression in the B16-OVA melanoma model were significantly reduced in response to RAW 264.7-derived exosome injections indicating their potential in immunotherapy. In the E.G7-OVA thymoma model, mice were initially injected with RAW 264.7-derived exosomes, before being challenged with the tumor cells in a cancer vaccine approach and they were proven to strongly prevent tumor establishment. Essentially, RAW 264.7-derived exosomes were confirmed to induce strong antigen-specific immune responses and presented a tumor reducing capacity, and thus provide an alternative method for exosomal loading for the development of novel cancer therapies.

4.1.4 Study IV

In the present study, we examined the feasibility to use the antibody-based PEA method for the investigation of exosomal protein levels and the ability to trace the exosome origin. Consequently, we analyzed protein content of several cell lines and their exosomes and additionally human body fluid-derived exosomes from healthy donors. We applied different protein panels related to cancer, cardiovascular diseases and inflammation to investigate their potential use for the analysis of exosomal protein content. We identified proteins that differed between cell lines and their corresponding exosomes, indicative of a selective loading of certain proteins into the exosomes. We also detected shared protein profiles, demonstrating traceability of exosomes back to their respective cell or tissue of origin, which is important for the use of exosomes as disease markers, where identifying their cellular source could be of importance. In addition to the use of cell lines and their originated vesicles, we also explored if human body fluid-derived exosomes, breast milk and seminal fluid, from healthy individuals could be analyzed using the PEA technology. Importantly, we found different protein profiles in the breast milk-derived exosomes compared to the prostate-derived exosomes, which is interesting from a biomarker perspective. This study demonstrates the feasibility of PEA in detecting proteins in human body fluid-derived exosomes. The high sensitivity and low sample requirement achieved by PCR amplification of the target proteins, promotes the use of PEA as a possible analytical and screening tool. Exosome samples contain a large number of proteins that may reflect the health status of the cells they originate from, this study successfully demonstrates their future potential as biomarkers in a clinical setting and that the PEA technology can be applied for this purpose.

4.2 Discussion

4.2.1 Antigen internalization and loading onto exosomes

The cellular uptake and processing of free antigens is well established, although the mechanism for how DCs indirectly load antigens onto exosomes is currently not fully known. Antigen processing might vary, and therefore the efficiency of antigen loading to exosomes might fluctuate. Antigen uptake may occur via the specific clathrin-mediated endocytosis, or by the unspecific micropinocytosis, dependent on cell type and the specific antigen (19). Free OVA is mainly taken up specifically via the mannose receptor expressed on DCs. Therefore, an

efficient antigen uptake is observed by BMDCs, which will prompt peptide processing and loading onto MHC class II molecules (205). Endosomal escape of the antigen and cross-presentation will facilitate the loading of peptides onto MHC class I molecules (20, 21, 206, 207). We performed indirect loading of BMDC-derived exosomes (study I and II) by which, the receptor-mediated antigen internalization accomplish loading of OVA to the exosomal surface, but may also lead to presence of antigen inside the exosomes which remains to be explored. In contrast, the RAW 264.7 cell line (study III) was not capable of efficient OVA uptake nor loading onto their exosomes. We have previously explored the ability to load OVA to other cell lines such as DC 2.4 and MuTuDC (unpublished data) with the overall aim to find cell lines suitable for antigen loading and exosome production in a larger scale to overcome batch variations. Regrettably, OVA presence was not identified on these cell line-derived exosomes.

Notably, our approach to load exosomes (study I and II) is limited to antigens that can be produced in high amounts, and therefore this loading strategy is not applicable to all antigens. Today, it remains to be further explored exactly how exosomes stimulate immune responses, and for this purpose, OVA still serves as a useful model in mice to follow antigen-specific immune responses *in vivo*. However, it is not known if the same mechanism for immune stimulation is applicable to all other antigens loaded to exosomes. We speculate that, at least partly, other antigens can be loaded to exosomes and induce similar effects *in vivo*, since exosomes themselves also provide costimulation important for inducing immune activation independently of the antigen-loaded. Individual antigens might also provide slightly different immune-stimulatory mechanisms depending on protein size, receptor specificity and loading efficiency onto the exosomes.

Furthermore, we also investigated if indirect loading of exosomes could be applied using other antigens. Here, we explored the loading of BMDC-derived exosomes using the hen egg-white lysozyme (HEL) shown to be processed by DCs. However, HEL uptake is not facilitated through specific receptor-mediated uptake, but solely rely on micropinocytosis, which offers a less efficient uptake (208, 209). We could verify the presence of HEL protein on our exosomes using both ELISA (data not shown) and western blot, although in limited amounts probably related to the indirect uptake (figure 8A). Another possibility could be that HEL is processed differently compared to OVA and are not loaded to exosomes in the same degree, which may partly be related to the protein size, intracellular processing mechanisms and peptide presentation, which were not assessed in the present study. We investigated the immune-stimulatory capacity of HEL loaded exosomes *in vivo*. Unfortunately, probably due to the limited antigen loading efficiency, we could not detect a sufficient IFN-γ secretion in response to splenocyte restimulation *ex vivo* using whole HEL protein, as most mice had IFN-γ levels similar to the controls (figure 8B). Moreover, no antigen-specific antibodies were detected in serum after injection of HEL loaded exosomes (data not shown). Interestingly, we observed an increased GC B cell response to the HEL loaded exosomes (figure 8C). Similar results, especially in response to unloaded exosomes, have been previously observed (210). We

speculate that this is caused by an unspecific immune activation towards an unknown antigen carried by the vesicles, e.g. fetal bovine albumin.

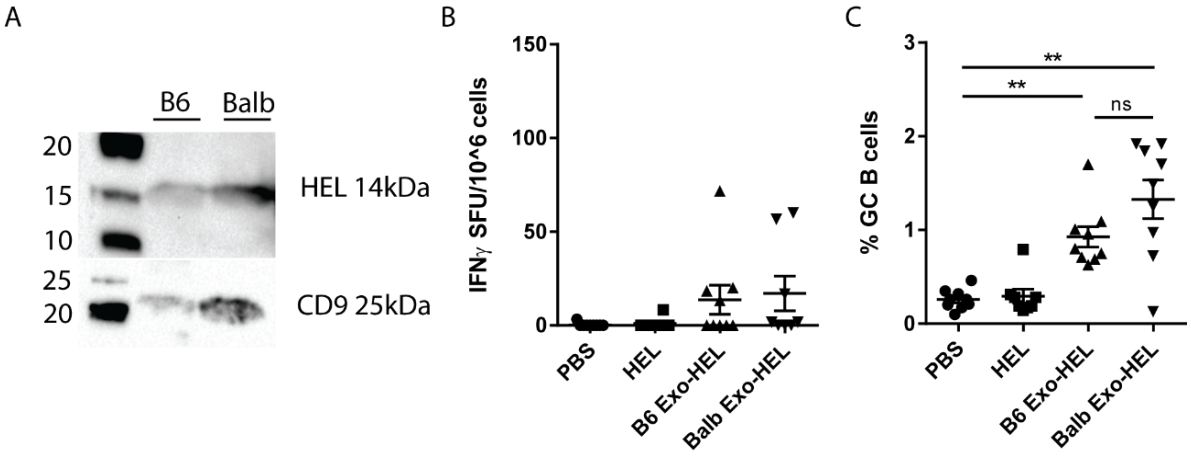

Figure 8. Indirect loading of BMDCs with HEL protein, followed by *in vivo* and *ex vivo* investigation of their immune-stimulatory potential. 40 µg exosomes PBS, free HEL, B6 exo-HEL or BALB/c exo-HEL injected i.v. in C57Bl/6 female mice. A) BMDC-derived exosomes carry HEL. B) IFN-γ secretion upon *ex vivo* restimulation of splenocytes. C) GC B cells after injection of HEL loaded exosomes. Data are pooled from 2 independent experiments, 9 mice/group.

These preliminary data suggest that it may be difficult to load other antigens to exosomes and an inefficient antigen loading limits the ability to study the exosomal immune-stimulatory capacity. More efficient loading strategies needs to be developed. Hence, lyophilization (study III) provides an alternative loading strategy, which is independent of cellular antigen uptake and reduces the amount of antigen required due to the high loading efficiency. Furthermore, lyophilization facilitates loading of adjuvants such as CpG-ODN for enhanced exosomal immune-stimulatory effect without changing the cell properties. Importantly, we show that exosomes loaded with CpG-ODN and treated with DNase are able to stimulate immune responses, proposing that CpG-ODN can be successfully loaded into the exosomes by lyophilization (study III). Loading of exosomes suggests a higher immune stimulation than if exosomes and stimuli/antigens would be co-administered. One possible explanation for this effect could be that vesicle carried OVA, CpG-ODN and αGC (study III) co-delivers the stimulatory signal to the same APC. Moreover, the ability to efficiently activate innate immunity by this technique is likely to enhance the properties of exosome-based vaccines. For cell fed antigens, the loading efficacy may be more batch dependent, which could be circumvented by the use of a standardized lyophilization procedure. Notably, alternative exosome loading, investigated by others, includes molecular engineering of cell lines for the expression of selected molecules for enhanced/specific immune activation and target cell specificity.

4.2.2 Exosomal uptake in vitro and in vivo

When comparing exosome T cell activation, varying observations are reported in the literature. T cell epitopes alone are sufficient in some studies and in others whole antigen or B cell epitopes are needed. T cell receptor affinity for the antigen presented by the MHC molecule

may vary in different models. Moreover, the exosomes themselves potentially also provide diverse costimulatory receptors and their activating capacity will also have an impact on the final conclusions drawn in different studies. For example, it has been demonstrated that DCs are crucial for the exosome immune activation *in vivo* (156) and *in vitro* for stimulation of T cells (152, 153, 157). Supporting the conclusion of exosome uptake *in vivo* by APCs, it has been demonstrated that exosomes are taken up by immature DCs for processing and peptide presentation on their MHC class II molecules (138). Furthermore, BMDC-derived exosomes primarily target the liver, followed by the spleen, gut and lungs *in vivo* in mice after i.v. injection (211). We observed that short after i.v. exosome injection MHC class II expression was upregulated on splenic DCs, macrophages and monocytes (study I). Moreover, we have previously shown that BMDC-derived exosomes bind or co-localize mainly with MZB, DCs and also to some extent with macrophages and FOB *in vitro*, however exosomes were not found associated with T cells (180). In study III, we observed that RAW 264.7-derived exosomes were mainly taken up by tissue resident macrophages and, to a smaller extent, by neutrophils and B cells after i.p. injection (data not shown). Taken together, this demonstrates that the injection route is important for the outcome of the studies performed, thus diverse results related to different injection routes have been discussed previously (212). Interestingly, in a recent study i.v. and i.p. injections were compared in tumor bearing mice, where i.v. injected DEX were shown slightly more efficient in reducing tumor growth (213). Moreover, our own *in vitro* data (study III), using the OT-I/Rag2$^{-/-}$ mice show that RAW 264.7-derived exosomes induce T cell proliferation in a splenocyte culture. Notably, due to different MHC background, the OVA transgenic CD8^{+} T cells are not able to respond directly to exosomes presenting the specific MHC class I peptide. This further suggests that also *in vitro*, DCs are likely responsible for exosome uptake and antigen presentation to T cells in our model, as these mice also lack functional B cells. In addition, adding CpG-ODN to the exosomes for *in vitro* stimulation further enhances proliferation suggesting the engagement of DCs (study III).

4.2.3 Exosomes in immunotherapy

Various immunotherapeutic strategies have been developed and tested in mouse models and in clinical trials, unfortunately we have not yet managed to develop therapies that are able to induce strong CTL responses in humans and the results observed in the mouse studies have not been fully reproducible in humans so far. Exosomes provide a cell-free alternative with some advantages compared to cells-based therapies, which support further investigation of exosomal-based immunotherapies. Especially, the use of DEX provide a prominent treatment option as they share immune-stimulatory characteristics with DCs however they can be produced in a larger scale and long-term storage is possible as exosomes are stable compare to cells. They also provide a longer half-life in circulation upon injection compared to their parental cell and are not prone to change characteristics due to the immune suppressive environment in the tumor. An efficient exosome-based anti-tumor immune therapy requires the activation of both innate and adaptive immune cells. In previous studies, we have shown that BMDC-derived exosomes can be loaded with antigens and that they are able to stimulate immune cells such as T cells both *in vitro* and *in vivo*. The rationale of using exosomes for

antigen transport is that the free antigens themselves are mostly not immunogenic, and most adjuvants developed to date are for viral diseases. We have repeatedly shown in our model that injection of free antigen in naïve mice will not trigger immune activation, suggesting that exosomes are important delivery vehicles for mounting an immune response towards the exosome carried antigens. Interestingly, we demonstrate in the long-term memory experiments that mice only respond to free OVA if they previously encountered the exosome carried antigen (study II). This finding suggests that the initial exosome injection potently stimulate naïve T cells. Then upon additional injections, antigen-specific T cells are present and therefore mice will respond to the free OVA.

4.2.3.1 Dendritic cell-derived exosomes in immunotherapy

In study I and II, BMDC-derived exosomes were investigated in mouse models for their immune-stimulatory role. Several clinical trials have been conducted, exploring peptide-loaded monocyte-derived dendritic cell exosomes or ascites-derived exosomes, respectively, in phase I clinical trials (175, 176, 181) and one phase II clinical trial (177). They were shown safe to administer however without inducing CTLs or presenting a sufficient tumor regression in the patients. The reason for the limited success in the trials applying DC-derived exosomes might be the use of peptides as mentioned earlier, but also related to the DC culture conditions for the generation of immune-stimulatory exosomes. The first trials used immature DC-derived exosomes followed by the most recent clinical trial which used IFN-γ matured DC-derived exosomes which appeared to be more potent immune stimulators. Also, the health status of the patients will affect the outcome of these trials, as they had progressed tumors when offered the treatment. Indeed, cancer patients are commonly immune suppressed, which limits the efficacy of exosomal treatment (35).

4.2.3.2 Allogeneic exosomes in immunotherapy

Stronger antigen-specific T cell responses have been observed in response to whole antigen-loaded compared to the peptide-loaded exosomes, both for the activation of $CD4^+$ T cells (160) and $CD8^+$ T cells (180), and both responses were dependent on B cells. The ability of BMDC-derived exosomes to stimulate T cells *in vivo* was enhanced in the presence of DCs, while *in vitro* exosomes alone were able to induce T cell proliferation (153, 160). We asked whether exosomal MHC/peptide complexes are important for antigen-specific immune responses *in vivo.* If this would be the case, exosomes would either interact directly with T cells or via cross-dressing of the MHC/peptide complexes for T cell stimulation (figure 9A and B). If not, the host DC would process the exosome/antigen (figure 9C), which would open up the possibility to use impersonalized exosome treatments. Therefore, we first studied the role of MHC class I molecules on exosomes by using MHC class I deficient mice, followed by exploring allogeneic exosomes in our tumor model (study I). We clearly demonstrated that MHC/peptide presentation was not important *in vivo* in the presence of whole antigen and the absence of MHC class I or MHC mismatch still provided tumor suppression. Allogenicity was further examined and we demonstrated a stronger short-term immune activation in response to two injections, however similar long-term responses in the tumor models were seen (study I and

II). Since then, allogeneic DEX have been further investigated for their tumor regressing capacity in a hepatocellular carcinoma mouse model. They were shown to influence the TME by enhancing IFN-γ and IL-2, inhibit IL-10 and TGF-β secretion and downregulate Tregs in the tumors (213). Thus, this study confirms our findings and further strengthen our hypothesis that allogeneic exosomes may work in cancer immunotherapy by processing of exosome carried antigens for peptide presentation on the host's own MHC molecules (figure 9C).

Moreover, $CD4^+$ T cell activation and Th1 cytokine secretion by exosomes were recently explored *in vitro* in an allogeneic setting (155), suggesting the use of allogeneic exosomes for the delivery of antigens. Of note, RAW 264.7-derived exosomes (study III) were also allogeneic, which strengthen the conclusions about the use of allogeneic exosomes in our models. Together all findings (study I-III) support the use of allogeneic exosomes as they demonstrate sufficient immune activation and tumor suppression. Moreover, allogenicity have been investigated in previous studies. For example, it has been shown that allogeneic exosomes are not able to directly activate T cells *in vivo* (159), although capable of delivering antigens for processing and presentation by MHC complexes on host DCs for activation of allo-reactive T cells (138). Notably, the ability to use allogeneic exosomes in therapies would provide an easily accessible and cost-beneficial approach.

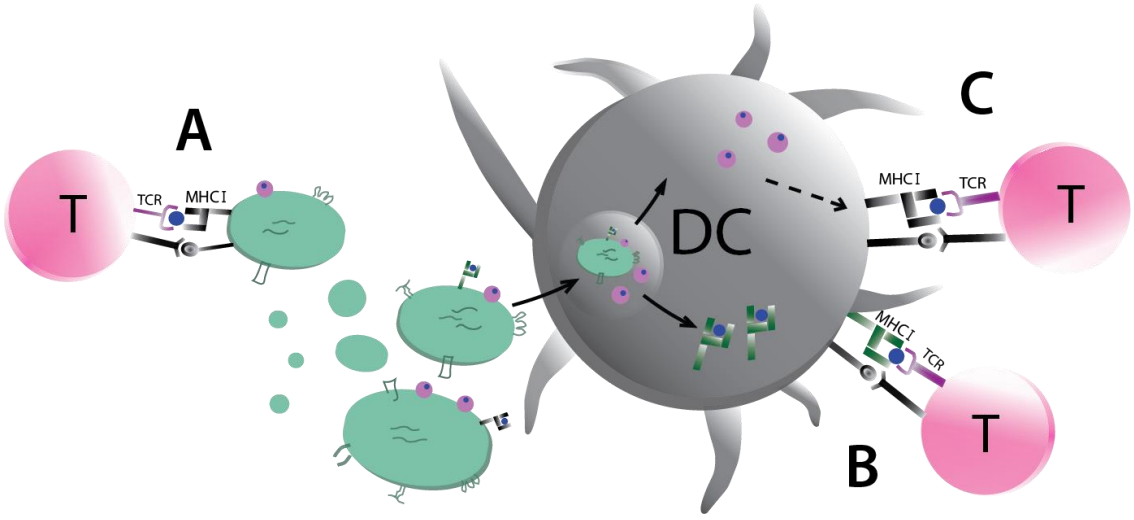

Figure 9. Potential exosome interaction and activation of T cells. A) Exosomes can directly interact with $CD8^+$ T cells for activation. B) Exosomes can be taken up by DCs and the MHC/peptide complex can be reused i.e. "cross-dressed" for $CD8^+$ T cell stimulation. C) Exosomes can be taken up and the exosome carried antigen can be processed for peptide presentation on the DCs own MHC complexes to activate $CD8^+$ T cells. Both scenario A and B require the presence of MHC class I expression on the exosomes, scenario C on the other hand, only require that the host have functional MHC molecules for antigen presentation. Thus, study I show that scenario C is most likely to occur *in vivo*.

4.2.3.3 Innate activation in the tumor model

DEX can activate the immune system in many different ways, for example by naturally loaded costimulatory molecules present on the exosomes and also additional antigens and adjuvants loaded onto the exosomes (study I-III). This thesis mainly focused on exploring the adaptive immune cells activated by exosomes. In our mouse models however we also see a strong effect

on tumor regression already within 2-3 days after BMDC-derived exosome injection, thus suggesting an innate immune stimulation. This may partly be induced by iNKT cell activation provided by the αGC delivered by the exosomes, which increase IFN-γ production and thereby also further stimulate an adaptive response i.e. effector cell upregulation. As discussed previously, the use of free αGC investigated in clinical trials induced iNKT cell anergy after the first injection (14, 52, 53), this was not observed when αGC was carried by the exosomes (182), and similar results have been observed for nanoparticles loaded with αGC. We observed a αGC stimulated iNKT cell proliferation, which is in line with previous findings (182). In addition, exosomes serve as natural adjuvants as they carry NK cell ligands and are able to stimulate innate immune responses and ADCC mediated NK cell killing of tumor cells, which is important for inhibiting tumor progression (178, 214). The survival of the mice could potentially be improved by the use of multiple injections to repeatedly boost the innate effector cells. In addition, we also explored the loading of the innate stimuli CpG-ODN onto exosomes for an improved immunogenicity. We found that CpG-ODN on exosomes strongly supported the anti-tumor immune responses in our model (study III). NK cell activation was also observed in the clinical trials performed using IFN-γ matured DEX. Many studies have focused on the induction of adaptive immune responses upon exosome injection. In order to mount potent adaptive responses, this also require strong initial innate responses. The role of innate versus adaptive immunity in our model remains to be explored in future studies.

4.2.3.4 Humoral responses to exosomal therapies

We explored the short-term *in vivo* response after a single exosome injection, and the experiments were terminated at day 7 before the induction of a strong humoral response (study I). This was followed by the investigation of antibodies in serum after repeated exosome injections, where we observed induction of antigen-specific antibodies (study II). Importantly, a ratio of IgG2c/IgG1 above 1 suggests a Th1 biased immune response, in accordance with previous findings (160, 182). This was induced by both syngeneic and allogeneic exosomes although the allogeneic exosomes were strongly enhancing the production of antigen-specific antibodies. This further strengthens the use of allogeneic exosomes in immune therapies against cancer. The antibody titers were followed over time for the comparison of syngeneic and allogeneic exosomes and to explore the long-term protection (study II). Importantly, the levels of total IgG were similar in all treatment groups (study I-II), suggesting that the antibody induction seen was related to a specific immune stimulation towards a specific antigen, and not an unspecific response. Moreover, we addressed the question whether allogeneic exosomes induced anti-MHC antibodies by mounting an immune response against the foreign MHC molecule present on the exosomes (study II). Indeed, our experiments detected serum antibodies specific towards the MHC class I molecules. Importantly, in serum from the long-term experiments, these antibodies were no longer detectable, suggesting a transient allo-recognition although no induction of long-term allogenicity (data not shown).

4.2.3.5 Improving exosome-based immunotherapy

We mainly focused on the role of antigen-specific $CD8^+$ T cell responses and iNKT cell engagement when using αGC on the exosomes (study I). The improved immune activation in response to αGC has been explored previously (182). We confirm the beneficial effect of αGC on the exosomes, and also show that exosomes lacking the MHC/peptide complex are functional in the presence of whole antigen. Future studies should aim at identifying tumor antigens that can be loaded onto exosomes in sufficient amounts, and development of methods that facilitates an efficient loading of antigens, such as shown for lyophilization (study III). Also, the use of allogeneic exosomes could open up the possibility to use transfected cell lines loaded with antigens which would provide a defined system for antigen loading onto the exosomes, such strategies are currently investigated. One example is the use of tumor cell line engineered to carry CpG-ODN on the exosomes, which were able to induce a strong anti-tumor effect *in vivo* (215). Moreover, combining exosomes with other immunotherapeutic strategies, such as immune-checkpoint inhibitors may improve their therapeutic effect. In fact, our preliminary data suggest that exosomes together with anti-CTLA-4 antibody treatment might provide a successful combination. However, this require further investigation. Taken together, several immunotherapeutic approaches are currently being tested in various combinations, as they will all hit diverse pathways of immune activation, their combined effect may be beneficial in the clinic. Also, since many of the currently available treatment options are associated with severe side effects, the use of suboptimal doses might be an alternative in combination with other treatments.

4.2.3.6 Tumor cell-derived exosomes in immunotherapy

The dual role of tumor cell-derived exosomes, both stimulatory and inhibitory, are described in the introduction. Thus, one can ask whether TEX provides a good treatment strategy in cancer immunotherapy as it is challenging to predict the outcome of such treatment due to different status/mutations of cancer cells. It has been suggested that the role of TEX in the TME is mainly to induce immune suppression. TEX are able to deliver tumor antigens to DCs and enhance their antigen presenting capacity, which would suggest the possibility to use TEX in cancer immunotherapy (171). When TEX are loaded to DCs, they may provide immune activation by providing costimulation, deliver tumor antigens to the DCs and by TEX selectively targeting MHC class II positive cells to activate $CD4^+$ T cells (163, 216).

4.2.3.7 Alternative immunotherapies

Passive immunotherapies like antibodies or T cells only induce weak immune responses and low memory T cell formation and therefore provides a limited vaccine effect. Instead, active therapies using DCs striving to achieve both $CD4^+$ and $CD8^+$ T cell activation, which drive memory and effector T cell formation, for improved vaccine properties. DC-based therapies have been extensively explored in clinical trials although struggling with some limitations, for example, DCs may change phenotype after injection due to the immune suppressive TME. Moreover, many cell-based vaccines produced are not of high enough quality to be reinfused into the patient.

4.2.4 Exosomes as diagnostic markers for disease

The use of exosomes as diagnostic markers for diseases have several advantages, for example various markers and protein patterns can be found in different combinations on the same exosome. The proteins carried by exosomes are concentrated after exosome isolation and are therefore measurable at higher levels compared to the levels observed in circulation, where specific proteins can also be masked. Moreover, exosomes may be traced to their cellular origin and provide information about cells or tissues without the need of invasive sampling. A lot of attention in the exosome field has focused on investigating exosomes as diagnostic tools due to their presence in different body fluids such as serum (91), plasma (101), milk (98), sputum (99) among others. Altered levels of exosomes and their cargo have been associated with a wide range of diseases, e.g. in bronchoalveolar lavage fluid of sarcoidosis patients (187), plasma and urine in cancer patients (100, 188), Alzheimer's disease (189) and cardiovascular diseases (190). The finding that exosomes carry mRNA and miRNA made them even more interesting from a biomarker perspective (121, 125, 170). The use of plasma-derived exosomes for biomarker discovery has been complicated due to the co-isolation of exosomes with the lipoproteins present in plasma. A recent study identified that SEC along with density gradient could serve as a possible method to eliminate the contaminating lipoproteins before performing proteomic analysis using mass spectrometry (217). In study IV, we focused on the use of exosomes for biomarker discovery exploring PEA as a sensitive tool to analyze the proteome of exosomes. Exosomal proteins have formerly been examined using multiple sources and methods (98, 144, 168, 194, 195, 218), thus many similarities in the proteins detected are shared with our present findings (study IV). The exosome field requires more sensitive proteomic methods as sample amounts often restricts the possibility to run a full proteomic analysis. The advantage with multiplex PEA compared to other available proteomic methods is the high specificity and sensitivity due to dual recognition via antibody-based detection of the proteins and signal amplification via PCR. In addition, the low sample volume requirement makes the method suitable for proteome analysis of exosomes (218-223). Furthermore, other screening methods are more time consuming, with limited reproducibility and not optimized for high-throughput analysis (224). We compared cell lines and their exosomes, and also body fluid-derived exosomes for examining the feasibility of using PEA on human exosome samples. We clearly demonstrate that PEA is useful in this aspect although struggling with the obvious limitations of being a biased method due to the selected antibodies used. Notably, the risk for false positive signals is limited since the two antibodies applied needs to be matched to hybridize and induce the PCR amplification, which have been carefully evaluated previously (225). Another disadvantage is the varying affinities of the antibodies therefore comparing different protein amounts is not achievable with the current setup. Importantly, due to the high sensitivity of PEA, this complicates the validation using other techniques. The next step in the aim of finding disease biomarkers would be to use PEA in a broader screening of patients and healthy individuals to identify exosomal protein patterns that can be linked to disease and/or prognosis.

4.2.5 Concluding remarks

The obstacles with cell-based therapies, previously emphasized, can be circumvented by the use of "off the shelf"-treatments such as exosomes. Exosome-based immunotherapy has been investigated in clinical trials although without a sufficient anti-tumor response, suggesting that the functional effects of exosomes need further investigation. The combination of several immune-stimulatory signals delivered by exosomes might potentiate their function as vaccines by the induction of both the innate and the adaptive immune responses. Moreover, impersonalized exosome immunotherapies would be of advantage by improving the treatment availability and lower the production cost. Notably, one limitation has been the need for an autologous system. However, it has been suggested that an allogenic system might work well without severe side effects (179). Our studies also support the beneficial use of allogeneic exosomes in immunotherapy.

Another suggested approach, not previously discussed, is to use exosomes as drug delivery vehicles. Exosomes have several properties that makes them interesting as target vesicles for drug delivery, e.g. they are small enough to cross the blood brain barrier and they target specific cells depending on their surface marker expression. In addition, exosomes can also reach distant targets with high specificity (226) and they are stable *in vivo* and *in vitro* (227). Moreover, novel exosome isolation methods are currently being developed, which will improve exosome research by providing ways to investigate purified exosomes from biological fluids and the possibility to select for certain vesicle populations. Currently available isolation methods restrict the ability to study individual exosomes and independent of the isolation method of choice, they all have their limitations. Furthermore, exosomes carry the potential to serve as biomarkers for disease. The advantage of applying exosomes as biomarkers are their noninvasive sampling due to their presence in body fluids, and also the accumulation of certain markers in the vesicles. Higher sensitivity in different approaches such as proteomics will increase the feasibility of using exosomes as biomarkers, and the ability to link the exosome phenotype to disease prognosis. At present, several clinical trials examining exosomes as biomarkers are currently ongoing, which will hopefully reveal potential exosomal biomarkers.

In conclusion, improved vaccination strategies might in the future lead to strong exosome-based cancer vaccine candidates. This requires development of novel technologies allowing efficient antigen loading of exosomes and further studies assessing the exosomal potential in clinical trials. Thus, this thesis suggests that allogeneic exosomes are a safe alternative treatment strategy and that they are potent inducers of immune responses. Moreover, this thesis also suggests a novel method for antigen loading of exosomes and also propose PEA as a highly sensitive method for exosomal proteomic analysis, which may be applied in future exosomal biomarker discoveries.

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"Det börjar som en fantasi, dagdrömmar om ett annat liv"

-Movits

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